INHIBITION OF TOXIC MATERIALS OR SUBSTANCES USING DENDRIMERS

FIELD OF THE INVENTION

This invention relates to inhibition of toxins and other toxic materials or substances, and in particular it relates to the use of dendrimers as binding agents for toxic peptides, proteins, or polyamines and other toxic materials or substances.

BACKGROUND OF THE INVENTION

Dendrimers are 3-dimensional polymeric materials of low polydispersity which are characterised by a large number of surface terminal groups. In addition the manner in which these materials are prepared allows tight control over the size, shape, and number and type of surface groups. Dendritic materials have several features that are useful for use as therapeutic materials: fixed shape which presents a large and defined surface with which to interact with biological surfaces and receptors; and the large number of terminal groups allow for multiple interactions with the biological targets.

International Patent Applications Nos. PCT/AU95/00350 (WO 95/34595) and PCT/AU97/00447 (WO 98/03573) disclose dendrimers such as a polyamidoamine or polylysine dendrimers having a plurality of terminal groups, wherein at least one of the terminal groups has an anionic- or cationic-containing moiety bonded or linked thereto. The contents of these published International patent applications are incorporated herein by reference.

The present invention provides the use of dendritic polymers in the inhibition of toxic materials or substances, including but not limited to toxins or toxic peptides such as snake, scorpion, spider and bee venoms, as well as toxic peptides or other toxic materials or substances released during bacterial or viral infection.

SUMMARY OF THE INVENTION

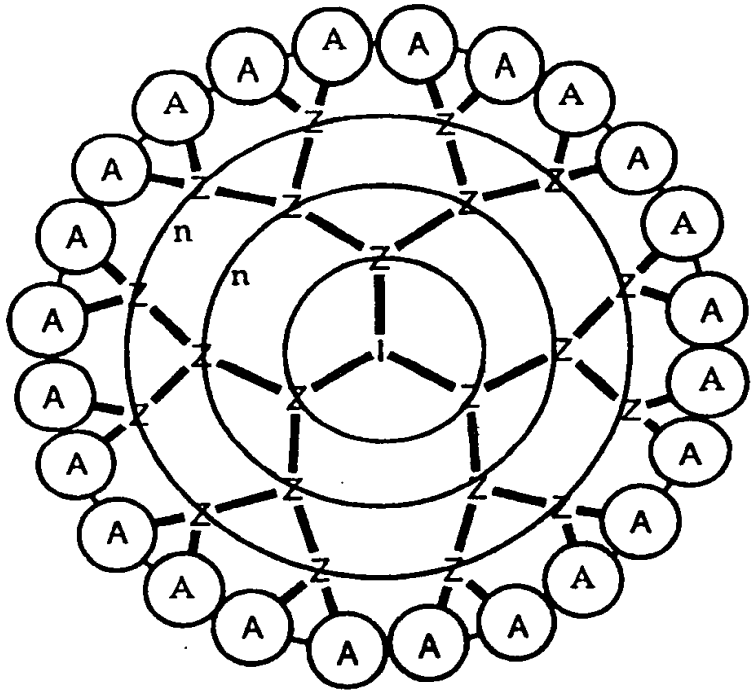
According to the present invention, there is provided a method of prophylactic or therapeutic inhibition of a toxic material or substance in a human or non-human animal patient, which comprises administration to the patient of an effective amount of a dendrimer having a plurality of terminal groups wherein at least one of said terminal groups has an anionic- or cationic-containing moiety bonded or linked thereto.

Particularly preferred compounds for use in the method of the present invention are dendrimers having sulfonic acid-containing moieties, carboxylic acid-containing moieties, phosphoric or phosphonic acid-containing moieties, boronic acid-containing moieties, neuraminic or sialic acid-containing moieties or moieties containing modified neuraminic or sialic acid; primary, secondary, tertiary or quaternary amino-containing moieties, pyridinium-containing moieties; guanidinium-containing moieties; amidinium-containing moieties; phenol-containing moieties; heterocycles possessing acidic or basic hydrogens; zwitterionic-containing moieties; or mixtures of the above moieties, linked to terminal groups thereof.

The compounds used in the method of this invention are referred to herein as polyionic dendrimers, and this term is used throughout this specification and the claims which follow to include not only the dendrimers *per se*, but also their pharmaceutically or veterinarily acceptable salts, for example the alkaline metal or alkaline earth metal salts such as the sodium, potassium or calcium salts, as well as pharmaceutically acceptable anions such as fluoride, chloride, bromide, iodide, citrate, acetate, p-toluene sulfonate, and the like.

DETAILED DESCRIPTION OF THE INVENTION

Preferred compounds used in accordance with the present invention include polyionic dendrimers of the general formula I:



I

wherein: I is an initiator core;

Z is an interior branching unit;

n is an integer which represents the number of generations of the dendrimer; and

A is an anionic- or cationic-containing moiety which may be linked to interior branching unit Z through an optional linking group X.

Dendrimers are macromolecular highly branched compounds formed by reiterative reaction sequences starting from an initial, core molecule with successive layers or stages being added in successive "generations" to build up a three-dimensional, highly ordered polymeric compound. Dendrimers are characterised by the following features: **I** an initiator core(I) which may have one or more reactive sites and be point-like or of significant size so as to effect the final topology of the dendrimer; **ii** layers of branched repeating units (Z) attached to the initiator core; **iii** functional terminal groups (such as moieties A) attached to the surface of the dendrimer, optionally through linking groups (such as linking groups X). The present invention uses dendritic structures as frameworks for the attachment of ionic moieties; the invention is not limited to the spherical dendrimers described in detail herein

but can be based on any dendritic structure. The variety of dendrimers in both shape and constitution are well known to persons skilled in the art.

The preparation of dendrimers is well known, and is described by way of example in U.S. Patents Nos. 4,289,872 and 4,410,688 (describing dendrimers based on layers of lysine units), as well as U.S. Patents Nos. 4,507,466, 4,558,120, 4,568,737 and 4,587,329 (describing dendrimers based on other units including polyamidoamine or PAMAM dendrimers). The dendrimers disclosed in these US patents are described as being suitable for uses such as surface modifying agents, as metal chelating agents, as demulsifiers or oil/water emulsions, wet strength agents in the manufacture of paper, and as agents for modifying viscosity in aqueous formulations such as paints. It is also suggested in U.S. Patents Nos. 4,289,872 and 4,410,688 that the dendrimers based on lysine units can be used as substrates for the preparation of pharmaceutical dosages.

International Patent Publications Nos. WO 88/01178, WO 88/01179 and WO 88/01180 disclose conjugates in which a dendrimer is conjugated or associated with another material such as a carried pharmaceutical or agricultural material. In addition, International Patent Publication No. WO 95/24221 discloses dendritic polymer conjugates composed of at least one dendrimer in association with a carrier material which can be a biological response modifier, and optionally a target director. These patent publications together with the U.S. patents mentioned above contain a broad disclosure of various dendrimers and processes for the preparation thereof, and the disclosure of each of these publications is incorporated herein by reference.

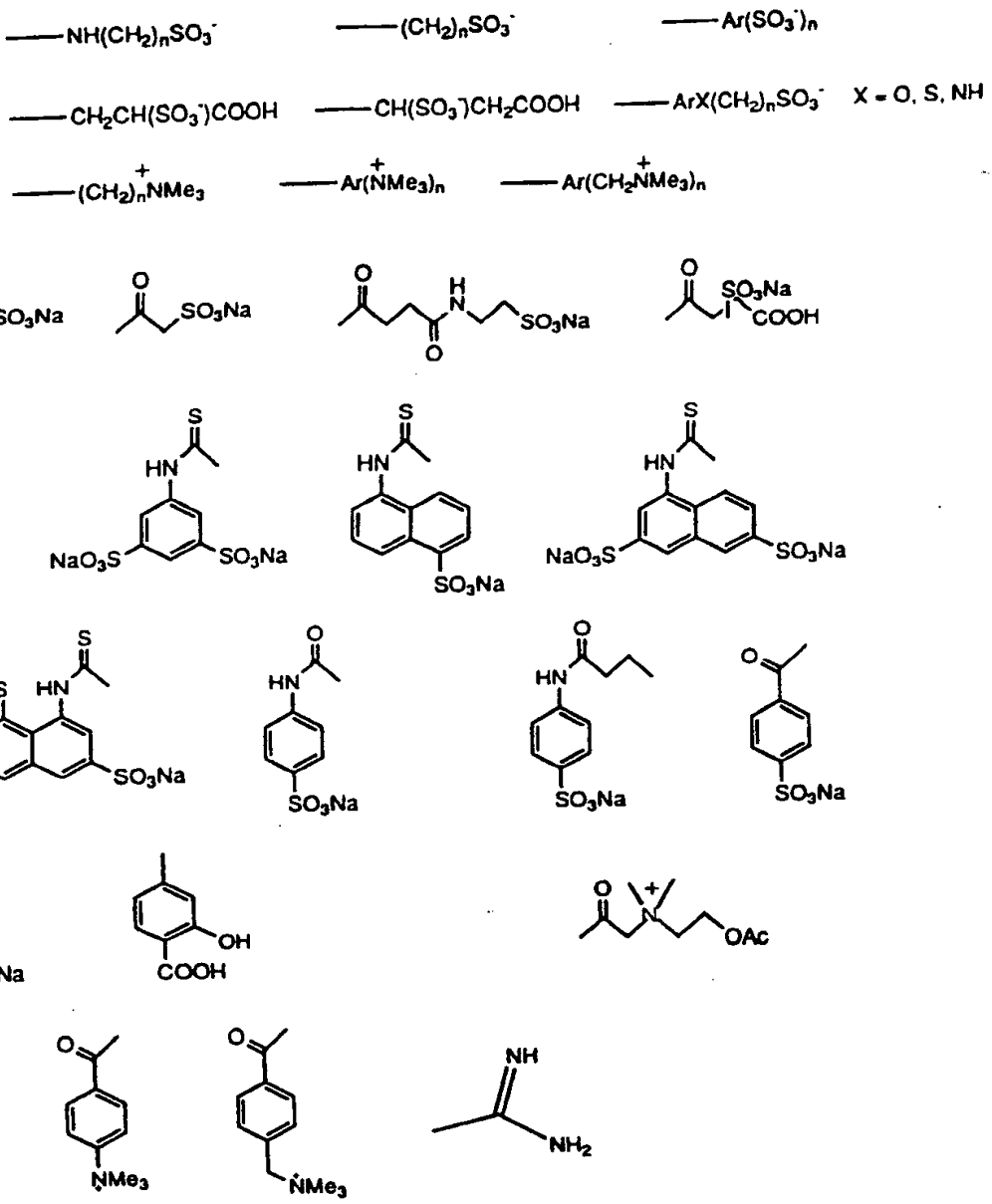
The term "dendrimer" as used herein is to be understood in its broadest sense, and to include within its scope all forms and compositions of these dendrimers as disclosed in Patent Publications Nos. WO 88/01178, WO 88/01179 and WO 88/01180. The term also includes linked or bridged dendrimers as disclosed in these patent publications.

The preferred dendrimers of the present invention comprise a polyvalent core

covalently bonded to at least two dendritic branches, and preferably extend through at least two generations. Particularly preferred dendrimers are polyamidoamine (PAMAM) dendrimers, PAMAM (EDA) dendrimers, poly(propyleneimine) (PPI) dendrimers and polylysine dendrimers.

In accordance with the present invention, at least one, and preferably a substantial number, of the terminal groups on the surface of the dendrimer has an anionic- or cationic-containing moiety covalently bonded thereto. The branches of the dendrimer may terminate in amino groups or other functional reactive groups such as OH, SH, or the like, which subsequently can be reacted with the anionic or cationic moieties. Where the terminal groups of the dendrimer are amine groups, the anionic- or cationic-containing moiety may be linked to the dendrimer by a variety of functional groups including amide and thiourea linkages. Preferred anionic- or cationic-containing moieties which may be bonded to the terminal groups of the dendrimer include sulfonic acid-containing moieties, carboxylic acid-containing moieties (including neuraminic and sialic acid-containing moieties and modified neuraminic and sialic acid-containing moieties), boronic acid-containing moieties, phosphoric and phosphonic acid-containing moieties (including esterified phosphoric and phosphonic acid-containing moieties) and primary, secondary, tertiary or quaternary amino-containing moieties, pyridinium-containing moieties; guanidinium-containing moieties; amidinium-containing moieties; phenol-containing moieties; heterocycles possessing acidic or basic hydrogens; zwitterionic-containing moieties; or mixtures of the above moieties.

Suitable anionic- and cationic-containing moieties which may be bonded or linked to the amino or other terminal groups include, by way of example, the following groups (in which n is zero or a positive integer, more particularly n is zero or an integer of from 1 to 20):

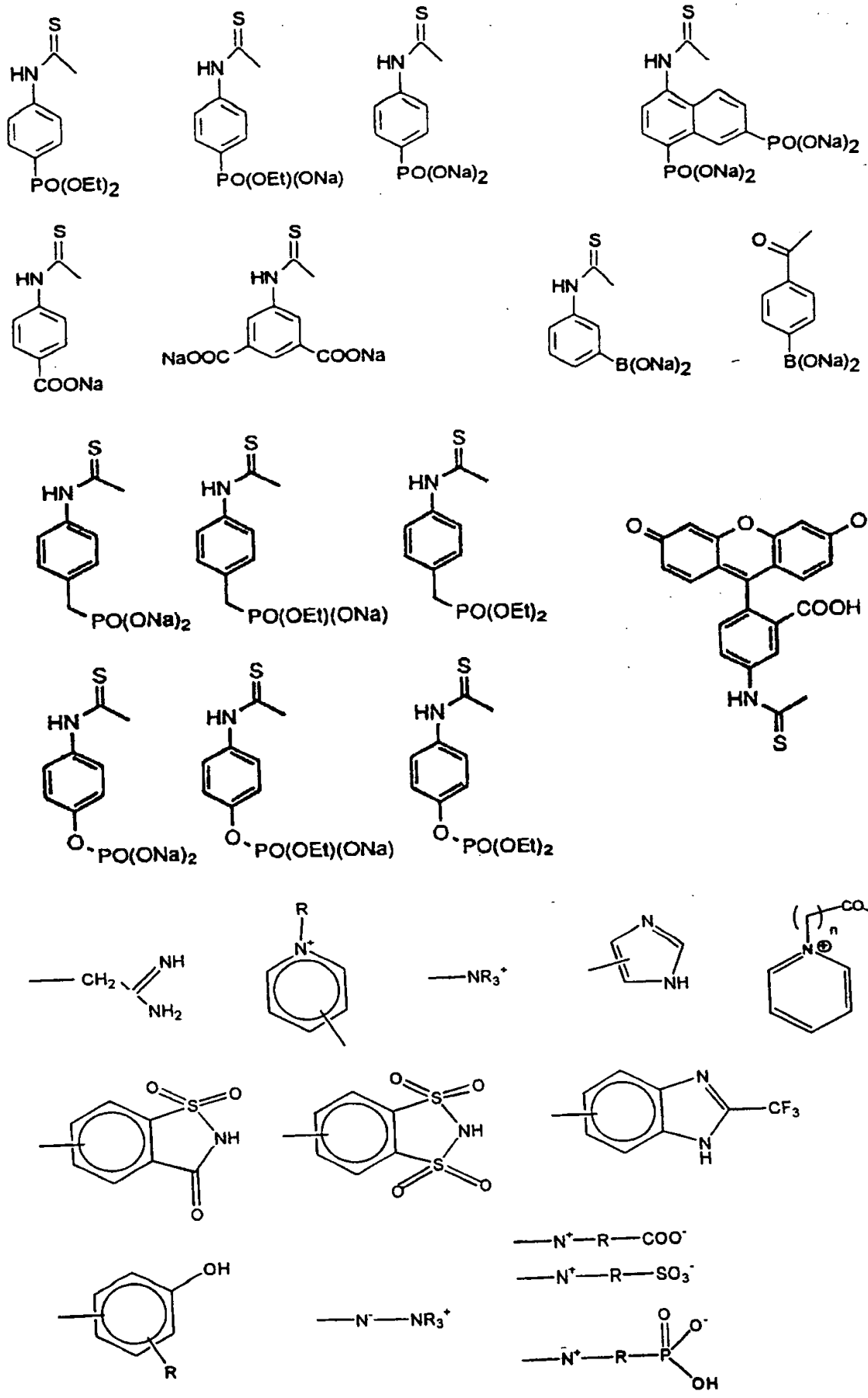


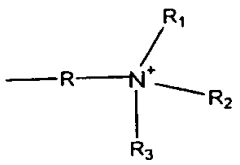
—ArXP(=O)(OR)$_2$ X=O, CH_2, CHF, CF_2 R=alkyl, aryl, H, Na.

—ArXP(=O)(OR^1)(NR^{2}R^3) X=O, CH_2, CHF, CF_2 R^1=alkyl, aryl, H, Na R^2, R^3=alkyl, aryl

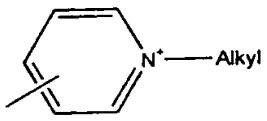
—Ar[P(=O)(OR)$_2$]$_n$ R=alkyl, aryl, H, Na n=1-3

—Ar[B(OH)$_2$]$_n$ n=1-3 —Ar[COOH]$_n$ n=1-3


S
HN
PO(OEt)2
PO(OEt)(ONa)
PO(ONa)2
COONa
NaOOC
B(ONa)2
O
OH
COOH
CH2
NH
NH2
NR3+
CO
n
CF3
R
N-
N+
COO-
SO3-
OH



R= alkyl or arylalkyl; R_1, R_2, R_3 (which may be same or different) = alkyl or arylalkyl



In addition to the above, various neuraminic or sialic acid-containing moieties or modified neuraminic or sialic acid-containing moieties may be bonded or linked to the dendrimers in accordance with this invention. These moieties include the various N- and O-substituted derivatives of neuraminic acid, particularly N- and O-acyl derivatives such as N-acetyl, O-acetyl and N-glycolyl derivatives, as well as moieties in which the neuraminic acid group is modified. Suitable modified neuraminic groups include groups which are substituted in the 4-position with an amino, amido, cyano, azide or guanidino group, as well as unsaturated neuramine acid groups. These moieties may be linked to the dendrimers through the 2-, 7-, 9- or 5-NAc positions.

Preferably, in the polyionic dendrimers of the general formula I, n is an integer of from 1 to 20 or more, more preferably from 1 to 10. Preferably also, the dendrimers include at least three or more terminal groups.

The optional linking groups which may be present to act as a spacer between the dendrimer and the moiety A, may consist of an alkyl chain (optionally substituted or branched), an alkoxy, polyalkoxy, alkylthio or polyalkylthio chain (optionally substituted), or an alkenyl, multiple alkenyl, alkynyl or multiple alkynyl chain (optionally substituted). Suitable spacer chains include groups of the formula $-(CH_2)_m-Z-(CH_2)_m-$, wherein Z is $-CH_2-$, -CH=CH-, -C≡C-, -O- or -S-, and m is an integer of from 1 to 15.

The anionic or cationic dendrimers of this invention may be prepared by standard

chemical methods which are well known to persons skilled in this art. Suitable methods are described by way of the example in Examples below.

As previously described, the anionic or cationic dendrimers of the present invention have been found to inhibit toxic materials or substances. The term "toxic materials or substances" as used herein is intended to refer in particular to toxins of biological (animal, plant, microbial or viral) origin, including but not limited to animal toxins or toxic peptides such as snake, scorpion, spider and bee venoms, toxic polyamines, and toxic peptides or other materials or substances released during bacterial infection (such as bacterial endotoxins and exotoxins), or during protozoal, fungal or viral infection.

The term "inhibition" is used herein in its broadest sense to include either full or partial inhibition or suppression of the toxic effect of the toxic material or substance in a human or non-human animal patient. The term is also used to encompass both prophylactic and therapeutic treatment.

Thus, in another aspect the present invention provides a pharmaceutical or veterinary composition for prophylactic or therapeutic inhibition of a toxic material or substance in a human or non-human animal patient, which comprises a dendrimer as broadly described above, in association with at least one pharmaceutically or veterinarily acceptable carrier or diluent.

The formulation of such compositions is well known to persons skilled in this field. Suitable pharmaceutically acceptable carriers and/or diluents include any and all conventional solvents, dispersion media, fillers, solid carriers, aqueous solutions, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art, and it is described, by way of example, in *Remington's Pharmaceutical Sciences*, 18th Edition, Mack Publishing Company, Pennsylvania, USA. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the

pharmaceutical compositions of the present invention is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

It is especially advantageous to formulate compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the human subjects to be treated; each unit containing a predetermined quantity of active ingredient calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier and/or diluent. The specifications for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active ingredient and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active ingredient for the particular treatment.

In yet another aspect, this invention provides the use of an effective amount of a dendrimer as broadly described above in the prophylactic or therapeutic treatment of, or in the manufacture of a medicament for prophylactic or therapeutic treatment of a human or non-human animal patient by inhibition of a toxic material or substance.

A variety of administration routes are available. The particular mode selected will depend, of course, upon the particular condition being treated and the dosage required for therapeutic efficacy. The methods of this invention, generally speaking, may be practised using any mode of administration that is medically acceptable, meaning any mode that produces therapeutic levels of the active component of the invention without causing clinically unacceptable adverse effects. Such modes of administration include oral, rectal, topical, nasal, inhalation, transdermal or parenteral (e.g. subcutaneous, intramuscular and intravenous) routes. Formulations for oral administration include discrete units such as capsules, tablets, lozenges and the like. Other routes include intrathecal administration directly into spinal fluid, direct introduction such as by various catheter and balloon angioplasty devices well known to those of ordinary skill in the art, and intraparenchymal injection into targeted areas.

The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing the active component into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the active component into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Compositions of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets, tablets or lozenges, each containing a predetermined amount of the active component, in liposomes or as a suspension in an aqueous liquor or non-aqueous liquid such as a syrup, an elixir, or an emulsion.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the active component which is preferably isotonic with the blood of the recipient. This aqueous preparation may be formulated according to known methods using those suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example as a solution in polyethylene glycol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono-or di-glycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

The active component may also be formulated for delivery in a system designed to administer the active component intranasally or by inhalation, for example as a finely dispersed aerosol spray containing the active component.

Other delivery systems can include sustained release delivery systems. Preferred sustained release delivery systems are those which can provide for release of the active

component of the invention in sustained release pellets or capsules. Many types of sustained release delivery systems are available. These include, but are not limited to: (a) erosional systems in which the active component is contained within a matrix, and (b) diffusional systems in which the active component permeates at a controlled rate through a polymer. In addition, a pump-based hardware delivery system can be used, some of which are adapted for implantation.

The active component is administered in prophylactically or therapeutically effective amounts. A prophylactically or therapeutically effective amount means that amount necessary at least partly to attain the desired effect, or to delay the onset of, inhibit the progression of, or halt altogether, the onset or progression of the particular condition being treated. Such amounts will depend, of course, on the particular condition being treated, the severity of the condition and individual patient parameters including age, physical condition, size, weight and concurrent treatment. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation. It is preferred generally that a maximum dose be used, that is, the highest safe dose according to sound medical judgement. It will be understood by those of ordinary skill in the art, however, that a lower dose or tolerable dose may be administered for medical reasons, psychological reasons or for virtually any other reasons.

Generally, daily oral doses of active component will be from about 0.01 mg/kg per day to 1000 mg/kg per day. Small doses (0.01-1 mg) may be administered initially, followed by increasing doses up to about 1000 mg/kg per day. In the event that the response in a subject is insufficient at such doses, even higher doses (or effective higher doses by a different, more localised delivery route) may be employed to the extent patient tolerance permits. Multiple doses per day are contemplated to achieve appropriate systemic levels of compounds.

The active component according to the invention may also be presented for use in the form of veterinary compositions, which may be prepared, for example, by methods that are

conventional in the art. Examples of such veterinary compositions include those adapted for:

(a) oral administration, external application, for example drenches (e.g. aqueous or non-aqueous solutions or suspensions); tablets or boluses; powders, granules or pellets for admixture with feed stuffs; pastes for application to the tongue;

(b) parenteral administration for example by subcutaneous, intramuscular or intravenous injection, e.g. as a sterile solution or suspension; or (when appropriate) by intramammary injection where a suspension or solution is introduced into the udder via the teat;

(c) topical application, e.g. as a cream, ointment or spray applied to the skin; or

(d) intravaginally, e.g. as a pessary, cream or foam.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

BRIEF DESCRIPTION OF THE DRAWINGS

In the accompanying drawings:

Figures 1 to 4 show the effects of various concentrations of BRI 2923 in inhibition of the HIV toxic Vpr peptide fraction P3.

Further features of the present invention will be apparent from the following Examples which are included by way of illustration, not limitation of the invention. In the following Examples, PAMAM dendrimers refer to polyamidoamine dendrimers based on an ammonia core as detailed in US Patents Nos. 4,507,466, 4,558,120, 4,568,737 and 4,587,329; PAMAM (EDA) dendrimers refer to polyamidoamine dendrimers based on an ethylene diamine core; and $BHAlys_x lys_y lys_z$ dendrimers refer to polylysine unsymmetrical

dendrimers based on a benzhydrylamine core and lysine branching units as described in US Patents Nos. 4,289,872 and 4,410,688. The polyamidoamine dendrimers PAMAM 1.0, PAMAM 2.0, PAMAM 3.0, PAMAM 4.0, PAMAM 5.0 or higher generation, PAMAM 4.0 (EDA), and the polylysine dendrimers $BHAlyslys_2$, $BHAlyslys_2lys_4$, $BHAlyslys_2lys_4lys_8$ and $BHAlyslys_2lys_4lys_8lys_{16}$, $BHAlyslys_2lys_4lys_8lys_{16}lys_{32}$, $BHAlyslys_2lys_4lys_8lys_{16}lys_{32}lys_{64}$, or higher generations prepared as described in US Patents Nos. 4289872, 4410688, 4507466, 4558120, 4568737 and 4578239 and International Patent Publications Nos. WO 88/01178, WO 88/01179, WO 88/01180 and WO 95/24221 referred to above.

EXAMPLE 1

Reaction of dendritic polymers with 2-acrylamido-2-methyl propane sulfonic acid to give sulfonic acid terminated dendrimers.

A PAMAM 1.0

Solid sodium carbonate (0.13g; 1.0mmol) was added slowly to a stirred solution of 2-acrylamido-2-methyl propane sulfonic acid (0.41g; 2.0mmol) in water (3ml). After the evolution of gas had ceased, the pH of the solution was 8.0. A solution of PAMAM 1.0 (0.12g; 0.33mmol) in water (1ml) was then added to the solution followed by the addition of four drops of a 40% aq. solution of benzyl trimethylammonium hydroxide. The solution was then heated under nitrogen at 60° for three days and then concentrated. The residue was purified by gel filtration (Sephadex G10; water) and then freeze dried to give the sulfonated PAMAM 1.0 dendrimer as an off white solid (0.51g). ^1H and ^{13}C nmr spectra showed a mixture of dialkylated and monoalkylated PAMAM 1.0 dendrimer (ca. 70:30). ^{13}C nmr (D_2O): δ 31.0, 31.1, 37.1, 37.7, 41.3, 48.6, 51.5, 53.1, 53.4, 55.6, 56.2, 61.2, 61.5, 178.3, 179.0, 179.8.

B PAMAM 2.0

PAMAM 2.0 was reacted with 2-acrylamido-2-methyl propane sulfonic acid as described above. The crude product was purified by gel filtration (Sephadex G10; water) and then freeze dried to give an off white solid. ^{1}H and ^{13}C nmr spectra showed a mixture of dialkylated and monoalkylated PAMAM 2.0 dendrimer (ca. 65:35). ^{13}C nmr (D_2O): δ 31.0, 31.1, 37.1, 37.7, 41.3, 48.7, 51.5, 53.4, 55.6, 56.2, 61.2, 61.5, 178.4, 179.0, 179.1, 179.6.

When the above reaction was repeated omitting the benzyltrimethylammonium hydroxide a similar result was obtained.

C PAMAM 3.0 **BRI2783**

PAMAM 3.0 was reacted with 2-acrylamido-2-methyl propane sulfonic acid as above except that a slight excess of sodium carbonate was used and the benzyltrimethylammonium hydroxide was omitted. ^{1}H and ^{13}C nmr spectra showed a mixture of dialkylated and monoalkylated PAMAM 3.0 dendrimer (ca. 50:50). ^{13}C nmr (D_2O): δ 31.0, 31.1, 36.9, 37.4, 41.1, 48.6, 51.5, 53.4, 55.7, 56.2, 61.1, 61.5, 178.2, 178.9, 179.0, 179.8.

D PAMAM 4.0 **BRI2784**

PAMAM 4.0 was reacted with 2-acrylamido-2-methyl propane sulfonic acid as described for PAMAM 3.0. ^{1}H and ^{13}C nmr spectra showed a mixture of dialkylated and monoalkylated PAMAM 4.0 dendrimer (ca. 35:65). ^{13}C nmr (D_2O): δ 31.0, 31.1, 36.9, 37.3, 41.1, 48.5, 51.5, 53.5, 55.7, 56.2, 61.1, 61.5, 178.1, 178.9, 179.0, 179.8.

EXAMPLE 2

Preparation of sodium sulfoacetamide terminated dendrimers.

A PAMAM 1.0

A solution of 4-nitrophenyl bromoacetate (0.40g; 1.5mmol) in dry DMF (1ml) was added to a stirred solution of PAMAM 1.0 (0.18g; 0.5mmol) in DMF (3ml). The resulting yellow solution was stirred for 20 hours at room temperature, when a ninhydrin test was negative. The solution was concentrated (30°/ 0.1mmHg) to give a yellow oil. This oil was partitioned between water and chloroform and the aqueous layer separated and washed with chloroform (2X) and finally with ethyl acetate. The aqueous solution was concentrated (35°/ 25mmHg) to give the bromoacetylated PAMAM 1.0 dendrimer as a yellow oil (0.36g;100%). ^{13}C nmr (D_2O): δ 32.8, 33.3, 43.0, 43.5, 54.4, 174.5, 176.4.

A solution of sodium sulfite (0.2g; 1.6mmol) in water (1ml) was added to a solution of the bromoacetylated PAMAM 1.0 dendrimer described above (0.36g; 0.5mmol) in water (5ml) and the solution left to stand at room temperature for eleven days. The yellow solution was concentrated to give a yellowish solid (0.60g). ^{13}C nmr (D_2O): δ 34.4, 43.1, 43.4, 54.0, 61.7, 171.3, 177.2.

The above reaction sequence could be carried out without isolating the bromoacetylated dendrimer by simply adding the sodium sulfite solution to the crude aqueous extract obtained from the first reaction.

B PAMAM 2.0

Method 1:

A solution of 4-nitrophenyl bromoacetate (0.18g; 0.7mmol) in dry DMF (1ml) was added to a stirred solution of PAMAM 2.0 (0.10g; 0.1mmol) in DMF (3ml). The resulting yellow solution was stirred for 20 hours at room temperature, when a

ninhydrin test was negative. The solution was then added with swirling to water (150ml) and the mixture extracted with chloroform (3X) and ethyl acetate. A solution of sodium sulfite (0.1g; 0.8mmol) in water (1ml) was added to the crude bromoacetylated dendrimer solution and the mixture allowed to stand for three days at room temperature. The yellowish solution was then concentrated to give a yellow solid residue, which was purified by gel filtration (Sephadex LH20; water) to give the sodium sulfoacetamide terminated PAMAM 2.0 dendrimer (103mg). ^{13}C nmr (D_2O): δ 33.0, 35.7, 36.0, 37.7, 40.3, 43.0, 43.2, 53.4, 53.7, 56.0, 61.6, 171.2, 174.6, 178.5.

Method 2:

Solid succinimidyl acetylthioacetate (67mg; 0.33mmol) was added to a solution of PAMAM 2.0 (52mg; 0.05mmol) in dry DMF (2ml) and the resulting solution stirred at room temperature for two days. The mixture was then concentrated ($30^{o}/10^{-3}$ mmHg) to give an oily residue. The residue was partitioned between water and chloroform, and the water layer separated and concentrated to give a viscous oil (117mg). ^{1}H and ^{13}C nmr showed the oil to be a mixture of the acylated dendrimer and N-hydroxy succinimide. Gel filtration (Sephadex G10; water) provide a pure sample of the acetylthioacetamide terminated PAMAM 2.0 dendrimer (29mg). ^{13}C nmr (D_2O): δ 34.0, 34.2, 37.3, 43.0, 43.1, 43.3, 53.5, 54.0, 56.3, 175.4, 177.2, 177.5.

A solution of the above functionalised dendrimer in 40% aqueous formic acid (7ml) was then added to an ice cold freshly prepared solution of performic acid (1.6mmol) in formic acid (2ml). The mixture was stirred for one hour at 0^{o} and then for twenty hours at room temperature. A small amount of activated charcoal was then added to decompose any excess peracid, the mixture stirred for 30 minutes then filtered and concentrated to give a viscous oil.

The crude product was dissolved in water, the pH adjusted to 9.0 with aqueous sodium bicarbonate and the material desalted by passage through a column of

Sephadex G10. A white solid (20mg;) was obtained after lyophylisation which was spectroscopically essentially the same as the material obtained by method 1. ^{13}C nmr (D_2O): δ 33.0, 38.7, 42.9, 43.0, 43.1, 53.9, 54.3, 56.5, 61.6, 171.2, 176.4, 177.0.

EXAMPLE 3

Preparation of sodium sulfosuccinamic acid terminated dendrimers

A PAMAM 1.0

Solid maleic anhydride (0.11g; 1.1mmol) was added to a stirred solution of PAMAM 1.0 (0.12g; 0.33mmol) in dry DMF (3ml). The mixture became a little warm and brownish as the anhydride dissolved and the resulting solution was stirred overnight at room temperature. The solution was then concentrated ($30^{o}/10^{-4}$mmHg) to give a viscous oil. ^{1}H and ^{13}C nmr (D_2O) showed complete conversion of the PAMAM 1.0 to the trisamide together with some maleic acid. ^{13}C nmr (D_2O): δ 33.1, 42.8, 43.1, 54.3, 135.0, 137.1, 169.1, 171.9, 173.3.

The crude trisamide was then dissolved in water (4ml) and solid sodium sulfite (0.20g; 1.6mmol) added. The resulting solution was allowed to stand at room temperature for four days and then concentrated. ^{1}H and ^{13}C nmr (D_2O) showed a 1:1 mixture of the regioisomeric sodium sulfosuccinamic acid terminated PAMAM 1.0 dendrimers together with some sulfosuccinic acid. The crude product was purified by gel filtration (Sephadex G10; water) to afford a sample of the sodium sulfosuccinamic acid terminated PAMAM 1.0 dendrimers (107mg). ^{13}C nmr (D_2O): δ 33.3, 39.6, 40.0, 42.9, 43.1, 54.0, 67.9, 69.4, 173.8, 176.3, 177.6, 181.8.

B PAMAM 2.0

A mixture of the regioisomeric sodium sulfosuccinamic acid terminated PAMAM 2.0 dendrimers was prepared as described above. ^{13}C nmr PAMAM 2.0 maleamic acid derivative (D_2O): δ 32.8, 33.0, 38.7, 42.9, 53.8, 54.3, 56.5, 135.2, 136.8, 169.2,

171.9, 173.5, 174.6. ^{13}C nmr PAMAM 2.0 sodium sulfosuccinamic acid derivatives (D_2O): δ 37.0, 40.1, 41.1, 43.0, 43.2, 43.9, 53.0, 53.3, 55.5, 68.0, 69.4, 173.8, 177.6, 179.1, 179.5, 179.8, 182.3.

C PAMAM 4.0 **BRI6038**

Solid maleic anhydride (60mg; 0.6mmol) was added to a stirred solution of PAMAM 4.0 (51mg; 0.01mmol) in dry DMF (2ml). The mixture initially became cloudy but soon gave a clear solution which was stirred overnight at room temperature. The solution was then concentrated (35°/10^{-4} mmHg) to give a viscous oil. ^{1}H and ^{13}C nmr (D_2O) showed complete conversion of the PAMAM 4.0 to the polyamide together with some maleic acid. The crude polyamide was then dissolved in water (2ml) and a solution of sodium sulfite (126mg; 1.0mmol) in water (2ml) added. The resulting solution was allowed to stand at room temperature for two days and then concentrated. ^{1}H and ^{13}C nmr (D_2O) showed a mixture of the regioisomeric sodium sulfosuccinamic acid terminated PAMAM 4.0 dendrimers together with some sulfosuccinic acid. The crude product was purified by gel filtration (Sephadex LH20; water) to afford a sample of PAMAM 4.0 terminated with 24 regioisomeric sulfosuccinamic acid groups (90mg). ^{1}H nmr (D_2O): δ 2.4-2.6; 2.7-3.1; 3.2-3.4; 3.9-4.0.^{13}C nmr (D_2O): δ 36.2; 39.8; 40.5; 43.0; 43.2; 53.5; 55.8; 68.1; 69.5; 173.8; 177.4; 177.6; 178.7; 182.3.

EXAMPLE 4

Preparation of sodium N-(2-sulfoethyl)succinamide terminated dendrimers

a Preparation of tetrabutylammonium N-(2-sulfoethyl)succinamic acid

Solid succinic anhydride (0.5g; 5.0mmol) was added to a stirred solution of tetrabutylammonium 2-aminoethylsulfonic acid (1.83g; 5.0mmol) in dry dichloromethane (30ml). The succinic anhydride slowly dissolved and the resulting cloudy solution was stirred overnight at room temperature. The mixture was filtered

and the filtrate concentrated to give a viscous oil (2.41g). ^{13}C nmr showed complete conversion to the desired monoamide together with a small amount of succinic acid. Repeated precipitation of the product by dropwise addition of a dichloromethane solution to a large excess of diethyl ether gave tetrabutylammonium N-(2-sulfoethyl)succinamic acid as a white solid (1.762g; 76%), mp 125-127°C. ^{1}H nmr ($CDCl_3$): δ 0.86 (t, 12h, $4xCH_3$), 1.28 (m, 8H, $4xCH_2$), 1.50 (m, 8H, $4xCH_2$), 2.33 (m, 2H, CH_2COOH), 2.44 (m, 2H, CH_2CONH), 2.76 (m, 2H, CH_2NHCO), 3.12 (m, 8H, $4xCH_2N$), 3.50 (m, 2H, $CH_2SO_3^-$), 7.53 (br t, 1H, NH). ^{13}C nmr ($CDCl_3$): δ 13.5 ,19.5, 23.8, 30.1, 30.9, 35.6, 50.0, 58.5, 172.0, 174.1.

b Preparation of tetrabutylammonium 4-nitrophenyl N-(2-sulfoethyl)succinamate

A solution of dicyclohexylcarbodiimide (45mg; 0.22mmol) in dry dichloromethane (1ml) was added to a stirred solution of tetrabutylammonium N-(2-sulfoethyl)succinamic acid (94mg; 0.20mmol) in dichloromethane (2ml), and the mixture stirred overnight at room temperature. The resulting suspension was filtered and the filtrate concentrated to give the crude active ester, which was used without further purification.

A Preparation of sodium N-(2-sulfoethyl)succinamide terminated PAMAM dendrimers

PAMAM 4.0 **BRI2786**

A solution of the crude tetrabutylammonium 4-nitrophenyl N-(2-sulfoethyl)succinamate (0.30mmol) in dry DMF (1ml) was added to a stirred solution of PAMAM 4.0 (51.5mg; 0.01mmol) dissolved in 50% aqueous DMF (3ml) and the resulting yellow solution stirred overnight at room temperature. The mixture was then concentrated ($35°/10^{-5}$ mmHg) and the yellow residue partitioned between water and chloroform. The water layer was separated, washed with chloroform (2X) and ethyl acetate, and then concentrated to give a yellow oil (134mg). The crude product was converted to the sodium salt by passage through a column of Amberlite IR 120(Na) to yield 85mg of material. This material was further purified by gel

filtration (Sephadex LH20; water) to give the sodium N-(2-sulfoethyl)succinamide terminated PAMAM 4.0 dendrimer (45mg). ^{13}C nmr (D_2O): δ 33.2, 33.6, 35.5, 39.0, 39.5, 42.8, 43.2, 53.8, 54.1, 54.4, 56.6, 176.5, 176.9, 177.2, 178.9, 179.4.

The corresponding PAMAM 1.0 and PAMAM 3.0 (**BRI2785**) dendrimers terminated with sodium N-(2-sulfoethyl)succinamide groups were similarly prepared.

^{13}C nmr PAMAM 3.0 derivative (D_2O): δ 33.4, 35.5, 39.0, 39.5, 42.9, 43.2, 53.8, 54.1, 54.3, 56.5, 176.4, 176.9, 177.4, 178.9, 179.4.
^{13}C nmr PAMAM 1.0 derivative (D_2O): δ 34.9, 35.5, 39.5, 42.9, 43.1, 53.7, 54.1, 179.0, 179.1, 179.3.

B Preparation of sodium N-(2-sulfoethyl)succinamide terminated polylysine dendrimers
$BHAlyslys_2lys_4lys_8lys_{16}$ **BRI2789**

Trifluoroacetic acid (1ml) was added to a suspension of $BHAlyslys_2lys_4lys_8DBL_{16}$ (36.5mg; 5.0μmol) in dry dichloromethane (1ml) and the resulting solution stirred at room temperature under nitrogen for two hours and then concentrated. The residue was dissolved in dry DMSO (2ml) and the pH adjusted to 8.5 with triethylamine. A solution of the crude tetrabutylammonium 4-nitrophenyl N-(2-sulfoethyl)succinamate (ca. 0.2mmol) in DMSO (1ml) was then added dropwise and the mixture stirred overnight at room temperature. The yellow solution was then concentrated ($50^{o}/10^{-5}$ mmHg) and the yellow residue partitioned between water and chloroform. The aqueous layer was separated, washed with chloroform (3X) and ethyl acetate, and then concentrated to give an oil (99mg). The crude product was converted to the sodium salt by passage through a column of Amberlite IR 120(Na) to yield 81mg of material. This material was further purified by gel filtration (Sephadex LH20; water) to give the sodium N-(2-sulfoethyl)succinamide terminated $BHAlyslys_2lys_4lys_8lys_{16}$ dendrimer (39mg). ^{13}C nmr (D_2O): δ 27.0, 32.3, 35.2,

35.3, 35.6, 35.7, 39.5, 43.5, 54.1, 58.5, 131.5, 132.0, 133.3, 145.1, 177.8, 178.0, 178.4, 178.8, 178.9, 179.2, 179.7, 179.8.

The corresponding $BHAlyslys_2$, $BHAlyslys_2lys_4$ (**BRI2787**) and $BHAlyslys_2lys_4lys_8$ (**BRI2788**) terminated with sodium N-(2-sulfoethyl)succinamide groups were similarly prepared.

^{13}C nmr $BHAlyslys_2lys_4lys_8$ derivative (D_2O): δ 26.9, 32.3, 35.1, 35.3, 35.6, 35.7, 39.5, 43.5, 54.1, 58.5, 131.6, 131.9, 132.2, 132.3, 133.2, 133.3, 145.0, 145.2, 177.2, 177.8, 177.9, 178.0, 178.2, 178.3, 178.6, 178.7, 178.8, 178.9, 179.2, 179.3, 179.7, 179.8.

^{13}C nmr $BHAlyslys_2lys_4$ derivative (D_2O): δ 26.9, 32.3, 35.1, 35.4, 35.7, 35.8, 39.5, 43.5, 54.1, 58.5, 61.8, 131.7, 132.0, 132.2, 132.3, 133.2, 133.3, 145.0, 145.1, 177.3, 178.0, 178.3, 178.4, 178.7, 178.9, 179.0, 179.3, 179.7, 179.8.

^{13}C nmr $BHAlyslys_2$ derivative (D_2O): δ 26.9, 27.1, 32.2, 32.3, 34.7, 34.8, 35.1, 35.3, 35.6, 35.7, 39.5, 43.4, 54.1, 58.6, 61.8, 131.7, 131.9, 132.2, 132.3, 133.3, 144.9, 145.0, 177.7, 178.4, 178.8, 179.0, 179.3, 180.0.

EXAMPLE 5

Preparation of sodium 4-sulfophenylthiourea terminated dendrimers

A PAMAM 4.0 **BRI2791**

Solid sodium 4-sulfophenylisothiocyanate monohydrate (500mg; 1.96mmol) was added to a solution of PAMAM 4.0 (300mg; 0.0582mmol) in water (10ml) and the resulting solution heated under nitrogen at 53° for two hours and then cooled. The solution was concentrated and the yellow solid residue purified by gel filtration (Sephadex LH20; water). The pure fractions were combined and freeze dried to give the sodium 4-sulfophenylthiourea terminated PAMAM 4.0 dendrimer as a fluffy white solid (370mg). ^1H nmr (D_2O) : δ 2.28; 2.52; 2.69; 3.15; 3.27; 3.60; 7.32 (d,

J=9Hz); 7.72 (d, J=9Hz). ^{13}C nmr (D_2O) : δ 36.9; 41.1; 43.1; 48.3; 53.6; 55.8; 129.0; 131.1; 144.4; 178.5; 179.1; 184.4.

The corresponding PAMAM 1.0, PAMAM 2.0 (**BRI2790**), PAMAM 3.0, and PAMAM 5.0 (**BRI2991**) dendrimers terminated with 3, 6, 12, and 48 sodium 4-sulfophenylthiourea groups respectively were similarly prepared.

B PAMAM 4.0 (EDA) **BRI6045**

Solid sodium 4-sulfophenylisothiocyanate monohydrate (130mg; 0.5mmol) was added to a solution of PAMAM 4.0 (EDA) (69mg; 0.01mmol) in water (4ml) and the resulting solution heated under nitrogen at 53° for two hours and then cooled. The solution was concentrated and the solid residue purified by gel filtration (Sephadex LH20; water). The pure fractions were combined and freeze dried to give PAMAM 4.0 terminated with 32 sodium 4-sulfophenylthiourea groups as a fluffy white solid (136mg). ^{1}H nmr (D_2O) : δ 2.30; 2.50; 2.70; 3.18; 3.62; 7.35 (d, J=9Hz); 7.72 (d, J=9Hz). ^{13}C nmr (D_2O) : δ 36.8; 41.0; 43.1; 48.4; 53.6; 55.7; 128.9; 131.0; 144.3; 178.5; 179.0; 184.5.

C BHAlyslys$_2$lys$_4$lys$_8$lys$_{16}$ **BRI2792**

Trifluoroacetic acid (4ml) was added to a suspension of BHAlyslys$_2$lys$_4$lys$_8$DBL$_{16}$ (0.73g; 0.1mmol) in dry dichloromethane (4ml) under nitrogen. A vigorous evolution of gas was observed for a short time and the resulting solution was stirred at room temperature for two hours and then concentrated. The residual syrup was dissolved in water (5ml), the solution passed through a column of Amberlite IRA-401(OH) and the filtrate concentrated to give BHAlyslys$_2$lys$_4$lys$_8$lys$_{16}$ as a viscous oil (0.49g). The oil was redissolved in water (5ml) and N,N-dimethyl-N-allylamine buffer (pH 9.5; 3ml) added. Solid sodium 4-sulfophenylisothiocyanate monohydrate (1.30g; 5.1mmol) was then added and the resulting solution heated under nitrogen at 53° for two hours and then cooled. The solution was concentrated and the brownish solid residue purified by gel filtration (Sephadex LH20; water). The pure fractions were

combined, passed through a column of Amberlite IR 120(Na) and freeze dried to give the sodium 4-sulfophenylthiourea terminated BHAlyslys$_2$lys$_4$lys$_8$lys$_{16}$ dendrimer as a fluffy white solid (374mg). ^{1}H nmr (D_2O) : δ 1.40; 1.72; 3.08; 3.42; 4.24; 4.60; 7.30; 7.40 (d, J=9Hz); 7.78 (d, J=9Hz). ^{13}C nmr (D_2O) : δ 27.3; 32.5; 35.9; 43.7; 48.9; 58.6; 63.3; 128.8; 131.0; 143.7; 144.7; 145.1; 177.7; 178.1; 183.8; 185.2.

~~The corresponding BHAlyslys$_2$lys$_4$lys$_8$, BHAlyslys$_2$lys$_4$lys$_8$lys$_{16}$lys$_{32}$ (BRI2992)~~, and BHAlyslys$_2$lys$_4$lys$_8$lys$_{16}$lys$_{32}$lys$_{64}$ (**BRI2993**) dendrimers terminated with 16, 64, and 128 sodium 4-sulfophenylthiourea groups respectively were similarly prepared.

EXAMPLE 6

Preparation of sodium 3,6-disulfonapthylthiourea terminated dendrimers

A PAMAM 4.0 **BRI2923**

Solid sodium 3,6-disulfonapthylisothiocyanate (160mg; 0.41mmol) was added to a solution of PAMAM 4.0 (51mg; 0.01mmol) in water (3ml) and the resulting solution heated under nitrogen at 53° for two hours and then cooled. The solution was concentrated and the brown solid residue purified by gel filtration (Sephadex LH20; water). The pure fractions were combined and concentrated to give the sodium 3,6-disulfonapthylthiourea terminated PAMAM 4.0 dendrimer as a brownish solid (73mg). ^{1}H nmr (D_2O) : δ 2.30; 2.60; 2.74; 3.20; 3.57; 7.75; 7.86; 8.28. ^{13}C nmr (D_2O) : δ 35.0; 39.9; 43.1; 48.1; 53.8; 56.1; 128.4; 128.6; 129.3; 131.0; 131.3; 136.0; 136.8; 138.2; 145.5; 146.0; 177.2; 177.8; 185.5.

The corresponding PAMAM 2.0 dendrimer terminated with sodium 3,6-disulfonapthylthiourea groups was similarly prepared.

B PAMAM 4.0 (EDA) **BRI6046**

Solid sodium 3,6-disulfonapthylisothiocyanate (220mg; 0.57mmol) was added to a solution of PAMAM 4.0 (EDA) (74mg; 0.01mmol) in water (4ml) and the resulting solution heated under nitrogen at 53° for two hours and then cooled. The solution was concentrated and the brownish solid residue purified by gel filtration (Sephadex LH20; water). The pure fractions were combined and concentrated to give PAMAM 4.0 terminated with 32 sodium 3,6-disulfonapthylthiourea groups as a tan solid (148mg). ^{1}H nmr (D_2O) : δ 2.30; 2.80; 3.20; 3.54; 7.74; 7.85; 8.25. ^{13}C nmr (D_2O) : δ 36.0; 40.8; 43.1; 48.3; 53.6; 55.9; 128.5; 129.4; 131.0; 131.3; 136.0; 136.8; 138.3; 145.5; 146.0; 178.2; 185.6.

C BHAlyslys$_2$lys$_4$lys$_8$lys$_{16}$ **BRI2999**

Trifluoroacetic acid (2ml) was added to a suspension of BHAlyslys$_2$lys$_4$lys$_8$DBL$_{16}$ (73mg; 0.01mmol) in dry dichloromethane (2ml) under nitrogen. A vigorous evolution of gas was observed for a short time and the resulting solution was stirred at room temperature for two hours and then concentrated. The residual syrup was dissolved in water (5ml), the solution passed through a column of Amberlite IRA-401(OH) and the filtrate concentrated to give BHAlyslys$_2$lys$_4$lys$_8$lys$_{16}$ as a viscous oil. The oil was redissolved in water (5ml) and N,N-dimethyl-N-allylamine buffer (pH 9.5; 3ml) added. Solid sodium 3,6-disulfonapthylisothiocyanate (234mg; 0.60mmol) was then added and the resulting solution heated under nitrogen at 53° for two hours and then cooled. The solution was concentrated and the brownish solid residue purified by gel filtration (Sephadex LH20; water). The pure fractions were combined, passed through a column of Amberlite IR 120(Na) and freeze dried to give BHAlyslys$_2$lys$_4$lys$_8$lys$_{16}$ terminated with 32 sodium 3,6-disulfonapthylthiourea groups as a fluffy off-white solid (119mg). ^{1}H nmr (D_2O) : δ 1.0-2.0; 3.18; 3.43; 4.31; 7.22; 7.80; 7.89; 8.25. ^{13}C nmr (D_2O) : δ 27.2; 32.4; 35.3; 43.7; 49.0; 58.5; 63.6; 128.4; 129.1; 131.4; 136.1; 136.6; 138.6; 139.0; 145.1; 145.6; 178.4; 184.8; 186.7.

EXAMPLE 7

Preparation of sodium 4-sulfonapthylthiourea terminated dendrimers

PAMAM 4.0 **BRI2997**

Solid sodium 4-sulfonapthylisothiocyanate (180mg; 0.5mmol) was added to a solution of PAMAM 4.0 (51mg; 0.01mmol) in water (5ml) and the mixture heated under nitrogen at 53° for two hours and then cooled. The water was distilled under reduced pressure from the resulting suspension and the off white solid residue purified by gel filtration (Sephadex LH20; water). The pure fractions were combined and freeze dried to give the sodium 4-sulfonapthylthiourea terminated PAMAM 4.0 dendrimer as a fluffy white solid (60mg). ^{1}H nmr (D_2O) : δ 2.20; 2.60; 3.14; 3.48; 7.23; 7.47; 7.56; 7.77; 7.93 (d, J=6Hz); 8.56 (d, J=6Hz). ^{13}C nmr (D_2O) : δ 35.8; 40.5; 43.1; 48.4; 53.6; 55.9; 127.6; 128.6; 130.3; 131.9; 132.5; 133.5; 134.7; 140.5; 142.7; 177.8; 178.0; 185.4.

EXAMPLE 8

Preparation of sodium 3,5-disulfophenylthiourea terminated dendrimers

PAMAM 4.0 **BRI6039**

Solid sodium 3,5-disulfophenylisothiocyanate (110mg; 0.32mmol) was added to a solution of PAMAM 4.0 (63mg; 0.012mmol) in water (3ml) and the resulting solution heated under nitrogen at 53° for two hours and then cooled. The solution was concentrated and the brownish solid residue purified by gel filtration (Sephadex G25; water). The pure fractions were combined and concentrated to give PAMAM 4.0 terminated with 24 sodium 3,5-disulfophenylthiourea groups as an off-white solid (110mg). ^{1}H nmr (D_2O) : δ 2.53; 3.08; 3.36; 3.66; 7.90; 7.95. ^{13}C nmr (D_2O) : δ 34.8; 41.0; 43.1; 48.0; 53.7; 56.2; 124.1; 128.6; 143.5; 148.8; 177.6; 185.0.

EXAMPLE 9

Preparation of sodium 3, 6, 8-trisulfonaphthylthiourea terminated dendrimers

PAMAM 4.0 **BRI2998**

Solid sodium 3, 6, 8-trisulfonaphthylisothiocyanate (250mg; 0.5mmol) was added to a solution of PAMAM 4.0 (51mg; 0.01mmol) and N,N-dimethyl-N-allylamine buffer (pH 9.5; 1ml) in water (2ml) and the mixture heated under nitrogen at 53° for two hours and then cooled. The mixture was concentrated under reduced pressure to give an orange solid. The residual solid was dissolved in water (2ml) and passed through a short column of Amberlite IR-120(Na). The filtrate was then concentrated and the residue purified by gel filtration (Sephadex LH20; water). The pure fractions were combined and freeze dried to give the sodium 3, 6, 8-trisulfonaphthylthiourea terminated PAMAM 4.0 dendrimer as an off-white solid (102mg). ^{1}H nmr (D_2O) : δ 2.65; 3.02; 3.30; 3.66; 8.05; 8.42; 8.59; 8.67. ^{13}C nmr (D_2O) : δ 33.2; 38.7; 43.2; 43.7; 47.8; 54.0; 54.3; 56.7; 131.0; 131.3; 131.9; 135.9; 138.0; 139.6; 143.8; 144.1; 145.6; 176.2; 176.5; 186.0.

The corresponding sodium 3,6,8-trisulfonaphthylthiourea terminated dendrimer BHAlys.$lys_2lys_4lys_8lys_{16}$ **BRI 7011** was prepared similarly.

EXAMPLE 10

Preparation of sodium 4-(sulfomethyl)benzamide terminated dendrimers

PAMAM 4.0 **BRI6040**

Solid 4-nitrophenyl 4-(chloromethyl)benzoate (200mg; 0.68mmol) was added to a stirred solution of PAMAM 4.0 (70mg; 0.014mmol) in dry DMSO (4ml) and the resulting yellow solution stirred at room temperature for two hours. The solution was then concentrated (10^{-4} mmHg; 40°) and the residue extracted with a mixture of water and dichloromethane (1:1). The remaining solid material was dissolved in DMSO (5ml) and a

solution of sodium sulfite (130mg; 1mmol) in water (3ml) added. The slightly cloudy mixture that resulted was left to stand for four days, after which time the addition of more water (2ml) resulted in the formation of a clear homogeneous yellow solution. The solution was then concentrated, first at 25mmHg and 40^{o} then at 10^{-4}mmHg and 50^{o} to give the crude product. The crude product was purified by gel filtration (Sephadex G25; water) to give PAMAM 4.0 terminated with 24 sodium 4-(sulfomethyl)benzamide groups (24mg). ^{1}H nmr (D_2O) : δ 2.25; 2.66; 3.08; 3.20; 3.33; 3.38; 4.01; 7.40 (br d); 7.62 (br d). ^{13}C nmr (D_2O) : δ 36.7; 40.9; 43.0; 43.6; 53.5; 55.5; 61.0; 131.6; 135.0; 137.2; 140.4; 174.5; 178.6; 179.2.

EXAMPLE 11

Preparation of 4-sulfobenzamide terminated dendrimers

PAMAM 4.0 (EDA) **BRI6116**

Solid potassium N-hydroxysuccinimidyl 4-sulfobenzoate (100mg; 0.3mmol) was added to a solution of PAMAM 4.0 (EDA) (35mg; 0.005mmol) in 0.1M pH 8.5 borate buffer (5ml) and the solution stirred at room temperature for two hours. The resulting milky solution at this stage had a pH of 4.5. 1M Sodium carbonate solution (1ml) was then added to give a clear solution which was concentrated to give the crude product as a white solid. The crude product was purified by gel filtration (Sephadex G25; water) to give PAMAM 4.0 (EDA) terminated with 32 sodium 4-sulfobenzamide groups (47mg). ^{1}H nmr (D_2O) : δ 2.25; 2.42; 2.63; 3.05; 3.18; 3.31; 3.38; 7.72 (d, J=8Hz); 7.78 (d, J=8Hz). ^{13}C nmr (D_2O) : δ 36.0; 40.4; 43.0; 43.7; 53.7; 55.8; 130.2; 132.2; 140.4; 150.1; 173.6; 178.0; 178.5.

EXAMPLE 12

Preparation of Sodium N-(4-sulfophenyl)propanamide terminated dendrimers

PAMAM 4.0 (EDA) **BRI6117**

Solid sodium N-(4-sulfophenyl)acrylamide (250mg; 1mmol) and solid sodium carbonate (106mg; 1mmol) were added successively to a stirred solution of PAMAM 4.0 (EDA) (78mg; 0.011mmol) in water (4ml). The resulting solution was stirred under nitrogen for four days and then freeze dried to give a fluffy white solid. The crude product was purified by gel filtration (Sephadex LH20; water to give PAMAM 4.0 (EDA) terminated with 64 sodium N-(4-sulfophenyl)propanamide groups (206mg). ^{13}C nmr showed a faint trace of what was taken to be mono alkylated terminal amino groups. ^{1}H nmr (D_2O) : δ 2.10; 2.48; 2.58; 2.79; 3.20; 7.42 (d, J=7Hz); 7.65 (d, J=7Hz). ^{13}C nmr (D_2O) : δ 36.5; 37.9; 41.1; 53.4; 55.6; 124.8; 130.9; 143.0; 144.2; 177.4; 178.5.

EXAMPLE 13

Preparation of Sodium 4-sulfophenylurea terminated dendrimers

PAMAM 4.0 (EDA) **BRI6115**

A solution of sodium sulfanilic acid (195mg; 1mmol) in dry DMSO (3ml) was added dropwise to a solution of N,N'- disuccinimidyl carbonate (530mg; 2mmol) in dry DMSO (4ml) and the resulting brownish solution stirred at room temperature for 20 hours. A solution of PAMAM 4.0 (EDA) (75mg; 0.011mmol) in dry DMSO (1ml) added and the solution stirred for a further 18 hours. The solution was then concentrated under high vacuum (10^{-5}mmHg; 35^{o}) to give a yellowish semi-solid. The crude product was dissolved in DMSO (4ml) and the solution added to 200ml of well stirred ethyl acetate. The precipitated white solid was collected by filtration and washed with ethyl acetate (2X) and ether (2X), then dried to give a white powder (275mg). This material was further purified by gel filtration (Sephadex LH20; water) to give PAMAM 4.0 (EDA) terminated with 32

sodium 4-sulfophenylurea groups (106mg). ^{1}H nmr (D_2O) : δ 2.31; 2.55; 2.75; 3.19; 7.32 (d, J=9Hz); 7.63 (d, J=9Hz). ^{13}C nmr (D_2O) : δ 36.3; 40.7; 43.3; 43.8; 53.7; 55.7; 123.3; 130.9; 140.9; 146.0; 161.4; 178.2; 178.6.

EXAMPLE 14

Preparation of N,N,N-trimethylglycinamide chloride terminated dendrimers

$BHAlyslys_2lys_4lys_8lys_{16}$ **BRI2922**

Trifluoroacetic acid (4ml) was added to a suspension of $BHAlyslys_2lys_4lys_8DBL_{16}$ (220mg; 30μmol) in dry dichloromethane (2ml) and the resulting solution stirred at room temperature under nitrogen for two hours and then concentrated. The residue was dissolved in dry DMSO (5ml) and the pH adjusted to 8.5 with triethylamine. Solid 4-nitrophenyl N,N,N-trimethylglycinate chloride (0.50g; 1.8mmol)was then added and the mixture stirred overnight at room temperature. The cloudy solution was then concentrated ($50^{o}/10^{-5}$ mmHg) and the residue partitioned between water and dichloromethane. The aqueous layer was separated, washed with dichloromethane (3X) and ethyl acetate, and then concentrated to give an oil (1.128g). The crude product was purified by gel filtration (Sephadex LH20; water) to give the N,N,N-trimethylglycinamide terminated $BHAlyslys_2lys_4lys_8lys_{16}$ dendrimer (116mg). ^{13}C nmr (D_2O): δ 25.5, 30.5, 30.8, 33.4, 42.1, 56.5, 57.1, 67.5, 68.1, 166.7, 167.0, 167.1, 176.0, 176.2.

EXAMPLE 15

Preparation of 4-Trimethylammoniumbenzamide terminated dendrimers

PAMAM 4.0 **BRI6043**

1,1'-Carbonyldiimidazole (85mg; 0.52mmol) was added to a solution of 4-trimethylammoniumbenzoic acid iodide (154mg; 0.5mmol) in dry DMF (4ml) and the mixture stirred at room temperature under argon for two hours. During this time a white

solid separated from the solution. A solution of PAMAM 4.0 (58mg; 0.011mmol) in dry DMF (2ml) was then added and the mixture stirred overnight at room temperature. After this time most of the precipitate had dissolved and a ninhydrin test of the solution was negative. The mixture was concentrated (10^{-4} mmHg; 30^{o}) to give a white solid residue. The crude product was purified by gel filtration (Sephadex LH20; 10% AcOH) to give PAMAM 4.0 terminated with 24 4-trimethylammoniumbenzamide groups as the acetic acid salt (89mg). ^{1}H nmr (D_2O) : δ 1.96; 2.65-2.85; 3.25-3.55; 3.64; 7.92. ^{13}C nmr (D_2O) : δ 25.8; 33.1; 33.5; 38.7; 43.1; 43.5; 53.5; 54.1; 56.4; 61.2; 124.8; 133.6; 139.9; 153.2; 173.2; 176.3; 176.8; 182.6.

The corresponding PAMAM 2.0 dendrimer terminated with 6 4-trimethylammonium benzamide groups was similarly prepared.

EXAMPLE 16

Preparation of 4-(Trimethylammoniummethyl)benzamide terminated dendrimers

PAMAM 4.0 **BRI6044**

Solid 4-nitrophenyl 4-(chloromethyl)benzoate (150mg; 0.5mmol) was added to a stirred solution of PAMAM 4.0 (52mg; 0.01mmol) in dry DMSO (3ml). The resulting yellow solution was stirred at room temperature for 20 hours, when a ninhydrin test was negative (pH ca.8.5). The solution was then concentrated (10^{-5} mmHg; 40^{o}) and the residue shaken with a mixture of water and dichloromethane (1:1). The insoluble gel-like material was collected by filtration, washed with water (2X) and dichloromethane (2X), and then air dried. The crude 4-(chloromethyl)-benzamide terminated dendrimer was dissolved in 25% aq. trimethylamine (20ml) and the yellow solution left to stand overnight. The solution was then concentrated, the residue dissolved in water (5ml) and the solution passed through a column of Amberlite IRA-401 (OH). The colourless filtrate was concentrated to give a viscous

oil which was purified by gel filtration (Sephadex G10; 10% AcOH) to give PAMAM 4.0 terminated with 24 4-(trimethylammoniummethyl)benzamide groups (90mg). ^{1}H nmr (D_2O) : δ 1.88; 2.65-2.80; 2.98; 3.10-3.60; 7.52 (br d, J=9Hz); 7.72 (br d, J=9Hz). ^{13}C nmr (D_2O) : δ 26.6; 33.4; 38.8; 43.2; 43.5; 53.6; 53.6; 54.1; 56.8; 62.8; 73.0; 132.1; 135.3; 137.5; 140.0; 176.4; 176.9; 183.6.

EXAMPLE 17

Preparation of N-(2-Acetoxyethyl)-N,N-(dimethylammonium)methyl-carboxamide terminated dendrimers

PAMAM 4.0

Solid 1,1'-carbonyldiimidazole (85mg; 0.52mmol) was added to a solution of N-(2-acetoxyethyl)-N-(carboxymethyl)-N,N-dimethylammonium bromide (135mg; 0.5mmol) in dry DMF (3ml) and the resulting solution stirred under nitrogen for two hours. A solution of PAMAM 4.0 (60mg; 0.012mmol) in DMF (2ml) was then added, which caused the immediate formation of a flocculant precipitate which slowly redissolved. The mixture was stirred for two days and then concentrated (10^{-4} mmHg; 40^{o}) to give a viscous oil. The crude product was purified by gel filtration (Sephadex G10; 10% AcOH) to give PAMAM 4.0 terminated with 24 N-(2-Acetoxyethyl)-N,N-(dimethylammonium)methylcarboxamide groups (64mg). ^{1}H nmr (D_2O) : δ 1.93; 2.05; 2.70; 3.10-3.60; 3.28; 3.93 (m); 4.14; 4.48 (m). ^{13}C nmr (D_2O) : δ 24.6; 26.2; 33.2; 38.7; 42.8; 42.9; 53.9; 57.4; 62.6; 67.3; 67.5; 168.9; 176.4; 176.8; 177.3; 183.2.

EXAMPLE 18

Preparation of Guanidino terminated dendrimers

PAMAM 4.0 **BRI6042**

A solution of PAMAM 4.0 (63mg; 0.012mmol) and methylthiopseudourea sulfate (170mg; 0.61mmol) in water (5ml) (pH 10.5) was heated under nitrogen at 80^{o} for two hours. The solution was then concentrated and the residue purified by gel filtration (Sephadex G10; 10% AcOH) to give PAMAM 4.0 terminated with 24 guanidino groups as the acetate salt (107mg). ^{1}H nmr (D_2O) : δ 2.00; 2.80 (br t); 3.09 (br t); 3.32; 3.45 (br t); 3.60 (br t). ^{13}C nmr (D_2O) : δ 25.2; 33.2; 33.4; 38.7; 41.2; 42.6; 43.4; 44.7; 53.5; 54.0; 56.3; 176.5; 176.7; 176.9; 181.6.

The corresponding PAMAM 2.0 dendrimer terminated with 6 guanidino groups was similarly prepared.

EXAMPLE 19

Preparation of 4-([1,4,8,11-tetraazacyclotetradecane]methyl)benzamide terminated dendrimers

PAMAM 4.0 **BRI6041**

A solution of 1-(4-carboxyphenyl)methyl-1,4,8,11-tetraazacyclotetradecane tetra hydrochloride (120mg; 0.25mmol), N-hydroxysuccinimide (60mg; 0.52mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (250mg; 1.3mmol) in pH 7 phosphate buffer (10ml) was allowed to stand a room temperature for one hour and then a solution of PAMAM 4.0 (32mg; 0.006mmol) in pH 7 phosphate buffer (10ml) added. The mixture was allowed to stand for two days and then concentrated. The residue was purified by gel filtration (Sephadex LH20; 10% AcOH) to give PAMAM 4.0 terminated with ca. 12 4-

([1,4,8,11-tetraazacyclotetradecane]methyl)-benzamide groups as determined by ^{1}H and ^{13}C nmr (80mg). The product was then dissolved in water and passed through a column of Amberlite IRA-401 (Cl) resin and then concentrated. The residue was dissolved in water (1ml), concentrated HCl (1ml) added, and the solution diluted with ethanol (30ml) to precipitate a white solid. The solid was collected by filtration (68mg). Once again ^{1}H and ^{13}C nmr showed ca. 50% functionalisation of the terminal amino groups. ^{1}H nmr (D_2O) : δ 2.17; 2.36; 2.50; 2.78; 2.85; 3.25; 3.40; 3.50; 3.60; 3.62; 4.49; 7.63 (br d); 7.78 (br d). ^{13}C nmr (D_2O) : δ 22.7; 23.1; 33.2; 38.8; 39.9; 40.2; 40.3; 41.0; 41.2; 42.0; 42.9; 43.2; 43.6; 45.5; 46.1; 49.1; 52.2; 53.9; 54.3; 56.6; 62.7; 132.5; 135.7; 137.1; 139.7; 174.3; 176.2; 176.3; 176.7; 177.0; 178.2; 178.5.

EXAMPLE 20

Preparation of 4-Carboxy-3-hydroxybenzylamine terminated dendrimers

PAMAM 4.0 (EDA) **BRI6119**

Sodium cyanoborohydride (32mg; 0.5mmol) was added to a mixture of PAMAM 4.0 (EDA) (69mg; 0.01mmol), 4-formyl-2-hydroxybenzoic acid (83mg; 0.5mmol), and sodium hydrogen carbonate (42mg; 0.5mmol) in water (4ml). The inhomogeneous orange mixture was stirred for four hours at room temperature, during which time it became homogeneous. The orange solution was then concentrated and the residue purified by gel filtration (Sephadex LH20; water) to give PAMAM 4.0 (EDA) terminated with ca. 32 4-carboxy-3-hydroxybenzylamine groups (91mg). ^{1}H and ^{13}C nmr (D_2O) shows mostly mono alkylation but with some signs of dialkylation of the terminal amino groups, both spectra show broad peaks. ^{13}C nmr (D_2O) :δ 37.0; 41.1; 50.9; 53.4; 55.5; 55.8; 61.5; 120.9; 122.2; 122.4; 132.3; 132.7; 135.0; 135.8; 163.5; 163.7; 169.0; 178.6; 179.3. ^{1}H nmr (D_2O) : δ 2.20; 2.35; 2.60; 3.15; 3.30; 3.55; 4.25; 6.68; 7.12; 7.55.

EXAMPLE 21

Preparation of 4-Carboxyphenylamide terminated dendrimers

PAMAM 4.0 (EDA)

Solid 4-carboxyphenylisothiocyanate (86mg; 0.48mmol) was added to a solution of PAMAM 4.0 (EDA) (69mg; 0.01mmol) in water (20ml). The pH of the resulting cloudy solution was adjusted to 9 with saturated $NaHCO_3$ solution and left to stir at room temperature for 24 hours. The reaction mixture was then filtered and the filtrate concentrated to give a white solid residue, which was purified by gel filtration (Sephadex LH20; water) and then freeze dried to give the product as a white fluffy solid (68mg).

EXAMPLE 22

Preparation of 3,5-Dicarboxyphenylamide terminated dendrimers

PAMAM 4.0 (EDA)

Solid 3,5-dicarboxyphenylisothiocyanate (112mg; 0.5mmol) was added to a solution of PAMAM 4.0 (EDA) (70mg; 0.01mmol) in water (5ml). The pH of the resulting cloudy solution was adjusted to 10 with 1M Na_2CO_3 solution and heated under nitrogen at 53° for 2 hours. The reaction mixture was then filtered and the filtrate concentrated to give a brownish solid residue, which was purified by gel filtration (Sephadex LH20; water) and then freeze dried to give the product as a pale brown solid (112mg).

EXAMPLE 23

Preparation of Sodium 4-Phosphonooxyphenylthiourea terminated dendrimers

PAMAM 4.0 (EDA)

Solid sodium 4-phosphonooxyphenylisothiocyanate (251mg) was added to a solution of PAMAM 4.0 (EDA) (69mg; 0.01mmol) in water (20ml). The resulting solution (pH 9) was stirted for 24 hours at room temperature under nitrogen. The reaction mixture was then concentrated to give a white solid residue, which was purified by gel filtration (Sephadex LH20; water) and then freeze dried to give the product as a fluffy white solid (86mg).

EXAMPLE 24

Preparation of Sodium 4-(Phosphonomethyl)phenylthiourea terminated dendrimers

PAMAM 4.0 (EDA)

Solid sodium 4-(phosphonomethyl)phenylisothiocyanate (97mg) was added to a solution of PAMAM 4.0 (EDA) (69mg; 0.01mmol) in water (30ml). The resulting solution was stirred for 3 days at room temperature under nitrogen, maintaining the pH at 8 with periodic addition of saturated $NaHCO_3$ solution. The reaction mixture was then concentrated to give a white solid residue, which was purified by gel filtration (Sephadex LH20; water) and then freeze dried to give the product as a fluffy white solid (102mg).

EXAMPLE 25

Preparation of Sodium Ethyl 4-(Phosphonomethyl)phenylthiourea terminated dendrimers

PAMAM 4.0 (EDA)

Solid sodium ethyl 4-(phosphonomethyl)phenylisothiocyanate (109mg) was added to a solution of PAMAM 4.0 (EDA) (69mg; 0.01mmol) in DMF (30ml). The resulting solution was stirred for 17 hours at room temperature under nitrogen, maintaining the pH at 8 with periodic addition of saturated $NaHCO_3$ solution. The reaction mixture was then concentrated to give a white solid residue, which was purified by gel filtration (Sephadex LH20; water) and then freeze dried to give the product as a fluffy white solid (30mg).

EXAMPLE 26

Preparation of C_n-alkyl linked 2-thiosialoside terminated dendrimers

Methyl [(8-octanoic acid N-hydroxysuccinimide ester) 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosid]onate was prepared by the following procedure.

To a solution of methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-2-S-acetyl-3,5-dideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosonate (Hasegawa *et al,* 1986) (100mg.) in dry dimethylformamide (1ml) was added 8-bromooctanoic acid (41mg.) and diethylamine (280mg.) and the solution stirred at 20° C for 17 hours. Solvent was removed under vacuum and the residue partitioned between ethyl acetate and ice cold 5% hydrochloric acid. The organic layer was washed with water, dried over sodium sulphate, and evaporated to give a residue (130mg.).This was dissolved in ethyl acetate (5ml.) and N-hydroxysuccinimide (26mg.) and

dicyclohexylcarbodiimide (46mg.) were added. The mixture was stirred at 20°C for 17 hours then the white precipitate was filtered off. The filtrate was concentrated and purified by flash chromatography on silica gel eluting with ethyl acetate. Fractions containing product were combined and evaporated to give a white foam 97mg. 71%.

Similarly were prepared:

Methyl [(11-undecanoic acid N-hydroxysuccinimide ester) 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosid]onate.

Methyl [(acetic acid N-hydroxysuccinimide ester) 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosid]onate.

Methyl [(4-butanoic acid N-hydroxysuccinimide ester) 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosid]onate.

Methyl [(4-methylbenzoic acid N-hydroxysuccinimide ester) 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosid]onate.

A PAMAM [EDA] 4.0 [(8-octanamido)- 5-acetamido-3,5-dideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosidoic acid]$_{32}$ **BRI 6112**

To a solution of the PAMAM [EDA] 4.0 (50mg.) in dry dimethyl sulphoxide(4ml.) under an inert atmosphere was added methyl [(8-octanoic acid N-hydroxysuccinimide ester) 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosid]onate(300mg.) and the solution stirred for 60 hours at 20°C. The solvent was removed under vacuum and the residue was dissolved in methanol (2ml.). This solution was subjected to size exclusion chromatography on Sephadex LH20

eluting with methanol. On evaporation of solvent, the product, PAMAM [EDA] 4.0 [methyl [(8-octanamido) 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosid]onate]$_{32}$ was obtained as a white powder. 182mg. 93%

This was converted to the free sialoside by the following method:

To a solution of PAMAM [EDA] 4.0 [methyl [(8-octanamido) 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosid]onate]$_{32}$ (182mg.) in dry methanol (3ml.) under argon at 20°C was added a freshly prepared 0.19M solution of sodium methoxide in methanol (7ml.) and the mixture stirred for 2.5 hours. The solvent was evaporated and the residue dissolved in water (10ml.) and stirred for 3 hours. This solution was subjected to size exclusion chromatography on Sephadex LH20 eluting with water. On lyophilisation, the product, PAMAM [EDA] 4.0 [(8-octanamido)- 5-acetamido-3,5-dideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosidoic acid]$_{32}$ was obtained as a pale lemon powder 110mg. 77%

By a similar procedure were prepared:

PAMAM [EDA] 4.0 [(11-undecanamido)-5-acetamido-3,5-dideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosidoic acid]$_{32}$ **BRI 6147**

PAMAM [EDA] 4.0 [(acetamido)- 5-acetamido-3,5-dideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosidoic acid]$_{32}$ **BRI 6121**

PAMAM [EDA] 4.0 [(4-methylbenzamido)- 5-acetamido-3,5-dideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosidoic acid]$_{32}$ BRI **6120**

B BHA lyslys$_2$lys$_4$lys$_8$lys$_{16}$ [(8-octanamido)- 5-acetamido-3,5-dideoxy-2-thio-

D-glycero-α-D-galacto-2-nonulopyranosidoic acid]$_{32}$ **BRI 6169**

A solution of BHA lyslys$_2$lys$_4$lys$_8$lys$_{16}$ (t-Boc)$_{32}$ (20.3mg.) in a mixture of trifluoroacetic acid (2ml.) and dichloromethane (2ml.) was stirred at 20°C for 2 hours then solvent was removed under vacuum. The residue was dissolved in dry dimethyl sulphoxide (1ml.) and di-isopropylethylamine (25mg.) and methyl [(8-octanoic acid N-hydroxysuccinimide ester) 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosid]onate (78mg.) were added. The mixture was stirred under argon at 20°C for 60 hours then solvent was removed under vacuum. The residue was dissolved in a freshly prepared 0.1M solution of sodium methoxide in methanol (2.5ml.) and the mixture stirred for 3 hours under argon at 20°C. The solvent was evaporated and the residue dissolved in water (1ml.) and stirred for 17 hours . This solution was subjected to size exclusion chromatography on Sephadex LH20 eluting with water. After lyophilisation ,the product, BHA lyslys$_2$lys$_4$lys$_8$lys$_{16}$ [(8-octanamido)- 5-acetamido-3,5-dideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosidoic acid]$_{32}$ was obtained as a white powder 44mg. 86%.

EXAMPLE 27

Preparation of dendritic sialosides modified in the 4-position of sialic acid

Methyl 4-azido-5-acetamido-7,8,9-tri-O-acetyl-2-S-acetyl-3,4,5-trideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosonate was prepared by the following procedure. To a solution of methyl 4-azido-5-acetamido-7,8,9-tri-O-acetyl-2-chloro-3,4,5-trideoxy-D-glycero-β-D-galacto-2-nonulopyranosonate (Sabesan, 1994) (5g.) in dry dichloromethane (150ml.) was added finely powdered potassium thiolacetate (5.8g.) and the suspension stirred vigorously at 20°C for 48 hours. The

mixture was filtered and evaporated to give a light brown foam (5.2g.). The required product was isolated by preparative reversed phase HPLC [C_{18}, 30% acetonitrile/water] as a white foam 3.9g. 72%.

Methyl [(8-octanoic acid N-hydroxysuccinimide ester) 4-azido-5-acetamido-7,8,9-tri-O-acetyl-3,4,5-trideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosid]onate was prepared by the following procedure.

To a solution of methyl 4-azido-5-acetamido-7,8,9-tri-O-acetyl-2-S-acetyl-3,4,5-trideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosonate (300mg.) in dry dimethylformamide (3.5ml.) was added 8-bromooctanoic acid (155mg.) and diethylamine (1.26ml.) and the solution stirred at 20° C for 17 hours. Solvent was removed under vacuum and the residue partitioned between ethyl acetate and ice cold 10% hydrochloric acid. The organic layer was washed with water, dried over sodium sulphate, and evaporated to give a yellow foam (385mg.).This was dissolved in ethyl acetate (20ml.) and N-hydroxysuccinimide (95mg.) and dicyclohexylcarbodiimide (175mg.) were added. The mixture was stirred at 20°C for 17 hours then the white precipitate was filtered off. The filtrate was concentrated and purified by preparative reversed phase HPLC [C_{18}, 30% acetonitrile/water] to give a white foam 340mg. 83%.

A PAMAM [EDA] 4.0 [(8-octanamido)- 4-azido-5-acetamido-3,4,5-trideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosidoic acid]$_{32}$ **BRI 6146**

To a solution of the PAMAM [EDA] 4.0 (72mg.) in dry dimethyl sulphoxide (5ml.) under an inert atmosphere was added methyl [(8-octanoic acid N-hydroxysuccinimide ester) 4-azido-5-acetamido-7,8,9-tri-O-acetyl-3,4,5-trideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosid]onate (318 mg) and the solution stirred for 60 hours at 20°C. The solvent was removed under vacuum and the residue was dissolved in methanol (2ml.). This

solution was subjected to size exclusion chromatography on Sephadex LH20 eluting with methanol. On evaporation of solvent, the product, PAMAM [EDA] 4.0 [methyl [(8-octanamido) 4-azido-5-acetamido-7,8,9-tri-O-acetyl-3,4,5-trideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosid]onate]$_{32}$ was obtained as a white foam. 225mg. 81%

The free sialoside was obtained by the following method:

To a solution of PAMAM [EDA] 4.0 [methyl [(8-octanamido) 4-azido-5-acetamido-7,8,9-tri-O-acetyl-3,4,5-trideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosid]onate]$_{32}$ (215mg.) in dry methanol (1ml.) under argon at 20°C was added a freshly prepared 1M solution of sodium methoxide in methanol (1ml.) and the mixture stirred for 3 hours. The solvent was evaporated and the residue dissolved in water (2ml.) and stirred for 17 hours. This solution was subjected to size exclusion chromatography on Sephadex LH20 eluting with water. On lyophilisation, the product, PAMAM [EDA] 4.0 [(8-octanamido)- 4-azido-5-acetamido-3,4,5-trideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosidoic acid]$_{32}$ was obtained as a fluffy white powder 160mg. 90%

B PAMAM [EDA] 4.0 [(8-octanamido)- 4-amino-5-acetamido-3,4,5-trideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosidoic acid]$_{32}$ **BRI 6149**

A slow steam of hydrogen sulphide gas was passed into a solution of PAMAM [EDA] 4.0 [(8-octanamido)- 4-azido-5-acetamido-3,4,5-trideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosidoic acid]$_{32}$ (25mg.) in a mixture of pyridine (40ml.) and water (20ml.) at 20°C for 5 days. The solution was then bubbled with nitrogen for 2 hours to remove excess hydrogen sulphide. The solution was evaporated to dryness and the residue taken up in water (5 ml) and filtered through a 0.45μm. membrane filter to

remove sulphur. On lyophilisation, the product, PAMAM [EDA] 4.0 [(8-octanamido)- 4-amino-5-acetamido-3,4,5-trideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosidoic acid]$_{32}$ was obtained as a fluffy white powder 23mg. 96%

EXAMPLE 28

Preparation of boronic acid terminated dendrimers.

4-Carboxyphenylboronic acid N-hydroxysuccinimide ester

To a solution of 4-carboxyphenylboronic acid (500mg.) in dry dimethyl formamide (5ml) were added N-hydroxysuccinimide (380mg.) and dicyclohexylcarbodiimide (680mg) The mixture was stirred at 20° C for 64 hours then the white precipitate was filtered off. The solvent was removed under vacuum and the residue dissolve in ethyl acetate (100ml.). This solution was washed with water, dried over sodium sulphate and evaporated to give a white solid which was crystallised from acetonitrile/water as fine needles 730mg. 92%

PAMAM [EDA] 4.0 [4-benzamidoboronic acid]$_{32}$ **BRI 6160**

To a solution of the PAMAM [EDA] 4.0 (69mg.) in dry dimethyl sulphoxide (5ml) under an inert atmosphere was added 4-carboxyphenylboronic acid N-hydroxysuccinimide ester (130mg.) and the solution stirred for 65 hours at 20°C. To the thick slurry was added 1M sodium carbonate solution (1ml.) and the clear solution stirred an additional 24 hours. The solvent was removed under vacuum and the residue was dissolved in 10% ammonia solution (5ml.). This solution was subjected to size exclusion chromatography on Sephadex LH20 eluting with 10% ammonia solution . On evaporation of solvent, the product, PAMAM [EDA] 4.0

[4-benzamidoboronic acid]$_{32}$ was obtained as a white fluffy solid. 110mg. 94%.

EXAMPLE 29

Preparation of Sodium 3,6-disulfonaphthylthiourea terminated dendrimers.

$BHAlyslys_2lys_4lys_8lys_{16}lys_{32}$

Trifluoroacetic acid (2ml) was added to a stirred suspension of $BHAlyslys_2lys_4lys_8lys_{16}DBL_{32}$ (147mg) in dry dichloromethane (2ml) and the resulting solution stirred at room temperature under nitrogen for two hours and then concentrated. The residue was dissolved in N,N-dimethyl-N-allylamine buffer (pH 9.5; 5ml) and then solid 3,6-disulfonaphthyl isothiocyanate (400mg) added. The pH of the mixture was then adjusted to 9.5 by the addition of 1M sodium carbonate and the solution heated at 53°C for three hours under nitrogen. The reaction mixture was concentrated and the residue redissolved in water and the solution passed through a column of Amberlite IR 120 (Na). The filtrate was concentrate was concentrated to give the crude product, which was purified by gel filtration (Sephadex LH20; water) to give $BHAlyslys_2lys_4lys_8lys_{16}lys_{32}$ with 64 sodium 3,6-disulfonaphthylurea groups as a white fluffy solid (175mg).

EXAMPLE 30

Preparation of Sodium 3,5-Disulfophenylthiourea terminated dendrimers.

$BHAlyslys_2lys_4lys_8lys_{16}lys_{32}$

Trifluoroacetic acid (3ml) was added to a stirred suspension of $BHAlyslys_2lys_4lys_8lys_{16}DBL_{32}$ (300mg; 0.02mmol) in dry dichloromethane (3ml) and the resulting solution stirred at room temperature under nitrogen for two hours and then concentrated. The residue was dissolved in water and the solution passed through a column of Amberlite IRA 401 (OH) and the filtrate concentrated to give a viscous oil (187mg). The oil was dissolved in a 1:1 mixture of pyridine/water (8ml) and solid sodium 3,5-disulfophenyl isothiocyanate (680mg; 2mmol) added.

The resulting solution was heated at 53°C for three hours under nitrogen. The solution was then concentrated to give a white solid residue. The crude product was purified by gel filtration (Sephadex LH20; water) to give $BHAlyslys_2lys_4lys_8lys_{16}lys_{32}$ with 64 sodium 3,6-disulfophenylurea groups as a white fluffy solid.

EXAMPLE 31

Preparation of Sodium 3,5-Dicarboxyphenylthiourea terminated dendrimers.

$BHAlyslys_2lys_4lys_8lys_{16}lys_{32}$ **BRI 6741**

Trifluoroacetic acid (3ml) was added to a stirred suspension of $BHAlyslys_2lys_4lys_8lys_{16}DBL_{32}$ (300mg; 0.02mmol) in dry dichloromethane (3ml) and the resulting solution stirred at room temperature under nitrogen for two hours and then concentrated. The residue was dissolved in water and the solution passed through a column of Amberlite IRA 401 (OH) and the filtrate concentrated to give a viscous oil (186mg). The oil was dissolved in a 1:1 mixture of pyridine/water (8ml) and sodium 3,5-dicarboxyphenyl isothiocyanate (450mg; 2mmol) added. The resulting solution was heated at 53°C for 13 hours under nitrogen. The solution was then concentrated to give a white solid residue. The crude product was purified by gel filtration (Sephadex LH20; water) to give $BHAlyslys_2lys_4lys_8lys_{16}lys_{32}$ with 64 sodium 3,6-dicarboxyphenylurea groups as a white fluffy solid.

The sodium 3,5-dicarboxyphenylthiourea terminated dendrimer PAMAM 4.0 (EDA) **BRI 6195** was similarly prepared.

EXAMPLE 32

Preparation of Sodium 4-phosphonooxyphenylthiourea terminated dendrimers.

$BHAlyslys_2lys_4lys_8lys_{16}lys_{32}$ **BRI 6181**

Trifluoroacetic acid (2ml) was added to a stirred suspension of $BHAlyslys_2lys_4lys_8lys_{16}DBL_{32}$ (147mg; 0.01 mmol) in dry dichloromethane (2ml) and the resulting solution stirred at room temperature under nitrogen for two hours and then concentrated to give a viscous oil. The oil was dissolved in N,N-dimethyl-N-allylamine buffer (pH 9.5; 5ml) and solid 4-phosphonooxyphenyl isothiocyanate (250mg) added. The pH of the resulting solution was adjusted to 10 with 1M sodium carbonate and the mixture heated at 53°C for three hours under nitrogen. The solution was then concentrated to give a white solid residue. The residue was redissolved in water and the solution passed through a column of Amberlite IR 120 (Na) and the filtrate concentrated. The residue was then purified by gel filtration (Sephadex LH20; water) to give $BHAlyslys_2lys_4lys_8lys_{16}lys_{32}$ with 64 sodium 4-phosphonooxyphenylurea groups as a white fluffy solid (150mg).

EXAMPLE 33

Preparation of Sodium 4-phosphonophenylthiourea terminated dendrimers.

$BHAlyslys_2lys_4lys_8lys_{16}lys_{32}$

Trifluoroacetic acid (2ml) was added to a stirred suspension of $BHAlyslys_2lys_4lys_8lys_{16}DBL_{32}$ (147mg; 0.01 mmol) in dry dichloromethane (2ml) and the resulting solution stirred at room temperature under nitrogen for two hours and then concentrated to give a viscous oil. The oil was dissolved in N,N-dimethyl-N-allylamine buffer (pH 9.5; 5ml) and solid 4-phosphonophenyl isothiocyanate (250mg) added. The pH of the resulting solution was adjusted to 9 with saturated sodium bicarbonate solution and the mixture heated at 53°C for

three hours under nitrogen. The solution was then concentrated to give a white solid residue. The residue was redissolved in water and the solution passed through a column of Amberlite IR 120 (Na) and the filtrate concentrated. The residue was then purified by gel filtration (Sephadex LH20; water) to give BHAlyslys$_2$lys$_4$lys$_8$lys$_{16}$lys$_{32}$ with 64 sodium 4-phosphonophenylurea groups **BRI 6196** as a white fluffy solid (152mg) after freeze drying.

EXAMPLE 34

Preparation of Sodium 4,6-diphosphononaphthylthiourea terminated dendrimers.

PAMAM 4.0

A solution of sodium 4,6-diphosphononaphthyl isothiocyanate (165mg) in water (2ml) was added to a solution of PAMAM 4.0 (51mg; 0.01mmol) in water (2ml). The pH of the mixture was adjusted to 9.5 with saturated sodium bicarbonate solution and the mixture vigorously stirred for one hour at room temperature and then heated at 53°C for three hours under nitrogen. The mixture was then filtered and the filtrate concentrated to give a brown solid residue. The crude product was purified by gel filtration (Sephadex G25; water) to give PAMAM 4.0 terminated with 24 sodium 4,6-diphosphononaphthylthiourea groups as a brown solid (81mg) after freeze drying.

EXAMPLE 35

Preparation of Fluoresceinthiourea terminated dendrimers.

PAMAM 4.0 (EDA)

Solid fluorescein isothiocyanate (188mg) was added to a solution of PAMAM 4.0 (EDA) (74mg; 0.01mmol) in water (3ml). Saturated sodium

bicarbonate solution was added to adjust the pH to 9 and the resulting homogenous solution stirred overnight at room temperature and then concentrated. The orange residue was purified by gel filtration (Sephadex LH20; water) to give PAMAM 4.0 (EDA) terminated with 21 fluoresceinthiourea groups as a fluffy orange solid (193mg) after freeze drying.

EXAMPLE 36

Preparation of Sodium (phenyl-3-boronic acid)-thiourea terminated dendrimers.

PAMAM 4.0 (EDA)

Solid (phenyl-3-boronic acid) isothiocyanate (100mg; 0.5mmol) was added to a solution of PAMAM 4.0 (EDA) (69mg; 0.01mmol) in water (5ml). 1M sodium carbonate was added to the isothiocyanate dissolved (pH ca.10). The mixture was then heated at 53°C for two hours under nitrogen, and then filtered and the filtrate concentrated to give a brownish solid residue. The crude product was purified by gel filtration (Sephadex LH20; water) to give PAMAM 4.0 (EDA) terminated with 32 (phenyl-3-boronic acid)thiourea groups as a white fluffy solid (87mg) after freeze drying.

EXAMPLE 37

Preparation of Pyridinium dodecyl carboxamido-terminated dendrimers.

PAMAM 2.0 dendrimer. **BRI-6807**

PAMAM generation 2.0 core (0.0479mmol; 50mg) was evaporated from a 0.5ml solution in MeOH and then re-dissolved in 10 ml of water. 1-N- pyridinium 12-dodecanoic acid bromide (0.14g; 0.384mmol), N-hydroxybenzotriazole hydrate [HOBT] (52mg; 0.384mmol) ; triethylamine (53μl 0.384mmol) and 1-(3-

diethylaminopropyl-3-ethyl) carbodiimide .HCl [EDC] (74mg; 0.384mmol), were added to the solution. This reaction mixture was stirred overnight at room temperature. The volume was reduced to a third under reduced pressure and the solution was chromatographed on a LH20 column using water as the eluent. Fractions containing the product were collected and pyridinium dodecylcarboxamide PAMAM 2.0 bromide isolated as a fluffy white solid by freeze drying.

^{1}H nmr (D_2O) : δ 1.15, 1.45, 1.9, 2.15, 2.75, 2.8, 3.15, 3.35, 3.5, 4.55, 8.05, 8.5, 8,8.

PAMAM 4.0 dendrimer. **BRI-6809**.

PAMAM generation 4.0 core (0.05mmol; 69mg) was evaporated from a 1.0ml solution in MeOH and then re-dissolved in 15 ml of water. 1-N- pyridinium 12-dodecanoic acid bromide (0.143g; 0. 4mmol), N-hydroxybenzotriazole hydrate [HOBT] (77mg; 0.4mmol) ; triethylamine (56μl 0. 4mmol) and 1-(3-diethylaminopropyl-3-ethyl carbodiimide .HCl [EDC] (77mg; 0.4mmol) were added to the solution.

This reaction mixture was stirred overnight at room temperature. The volume was reduced to a third under reduced pressure and the solution was chromatographed on a LH20 column using 1% triethylamine in water as the eluent. Fractions containing the product were collected and the pyridinium dodecylcarboxamide PAMAM 4.0 bromide was isolated as fluffy white solid by freeze drying. A small amount of the product was reacted with acetic anhydride to confirm the complete capping of the NH_2 end groups of the dendrimer core.

^{1}H nmr (D_2O) : δ 1.10, 1.45, 1.9, 2.1, 2.30, 2.5, 2.7, 3.2, 4.5, 8.00, 8.45, 8.80.

EXAMPLE 38

Preparation of saccharin-terminated dendrimers.

PAMAM 4.0 Dendrimer **BRI-6157**

To a solution of ethylenediamine core PAMAM 4.0 dendrimer core (275mg; 39.8uM) in dry dimethyl formamide (25ml) was added 6-(benzosulfimido) isothiocyanate (400mg; 1.67mM) and the mixture stirred at room temperature for 24 h. The cloudy solution was clarified by the adjustment of the pH with sodium carbonate solution to pH10-10.5. This solution was stirred for a further 24 h and volatiles removed on a rotary evaporator. The solution was chromatographed on a large Sephadex LH20 column and front fraction collected. The remaining fractions were collected and re-chromatographed on a smaller column. The combined front fractions were evaporated and freeze dried to yield the saccharin-terminated dendrimer product (450mg; 78%) as a fluffy white solid.

^{1}H nmr (D_2O) : δ 2.20, 2.50 3.23, 3.46, 3.63, 7.52, 7.87.

The saccharin-terminated BHA.Lys.$Lys_2Lys_4.Lys_8.Lys_{16}.Lys_{32}$... dendrimer **BRI-6189** was similarly prepared.

EXAMPLE 39

Inhibition of Cobra Venom and Bee Venom Toxin.

A Materials and Methods

Cytosensor Microphysiometer Protocol

The Cytosensor Microphysiometer (Molecular Devices Inc., CA) is a light addressable potentiometric sensor-based device that can be used to indirectly measure the metabolic rate of cells *in vitro* (Parce *et al.*, 1989; McConnell *et al.*,

1992). Metabolism is determined by measuring the rate of acid metabolite production from cells immobilised inside a microvolume flow chamber.

Human CEM cells were centrifuged and resuspended in low-buffered serum-free/bicarbonate-free RPMI 1640 medium (Molecular Devices; hereafter referred to as "modified medium"). The cells were seeded at a density of 60,000-75,000 cells/capsule onto the polycarbonate membrane (3 μm porosity) of cell capsule cups (Molecular Devices). Cells were immobilised using an agarose entrapment medium (Molecular Devices). The seeded capsule cups were transferred to sensor chambers containing the silicon sensor which detects changes in pH (and thus cellular metabolism). The Cytosensor system used for this set of experiments contained eight separate chambers for the measurement of acidification rates. Modified media was pumped across the cells at a rate of 100-120 μl/min. Each cell chamber was served by fluid from either of two reservoirs.

To measure the acidification rate, flow of the modified media was periodically interrupted, allowing the accumulation of excreted acid metabolites (lactic acid and CO_2). In this set of experiments, flow was stopped for 30s, during which time, a least squares fit slope to the change in voltage signal over time, the acidification rate (measured as μ V/s), was calculated. This rate data was normalised (using the 4-5 rate points prior to addition of compound) to allow direct comparison of the signals from the four chambers. Measurements of the acidification rate were made every 2 min. The chamber was held at 37°C.

Basal acidification rates were monitored (in the absence of any treatment) for at least 30 min. After this time, the venoms/peptides were exposed to the cells at a range of concentrations for periods of up to 4hrs. A concentration of toxin showing a pronounced effect on the cells, but less than maximal, was selected for testing of inhibition of this toxicity by a range of concentrations of BRI2923. In all

experiments, at least one chamber was not exposed to any of the compounds, providing a negative control.

BRI 2923 was dissolved in water and the solution was pH adjusted to 7.2. Concentrations ranging from 100 μM to 1 nM were added to the venom/media solutions and incubated for periods ranging from 6 min (the minimum incubation period possible using this equipment) to 1 hr and then introduced to the cells. All experiments were repeated in triplicate.

B Results

BRI2923 Inhibition of Cobra Venom.

Crude venom from the forest cobra (*Naja malenoleuca*) was tested. Cobra venom added to CEM cells caused an initial increase in cellular metabolism followed by cell death (cell lysis). Cobra venom was particularly damaging to the cells causing an initial increase in metabolism of approx. 80% followed rapidly by 100% cell death within the first 10 minutes. The venoms were initially tested at 10, 50 and 100 μg/ml. The submaximal response from 50 μg/ml was selected as the test dose in most experiments. Two concentrations of BRI2923 (10^{-4}M and 10^{-5}M) were used.

At all incubation periods (6, 30 and 60 mins), 10^{-5}M BRI2923 incubated with 50 μg/ml venom, reduced the initial increase in metabolic rate from approx. 80% to approx. 5% and delayed the onset of cell death by approx. 15-20 minutes.

10^{-4}M BRI2923, incubated with 50 μg/ml venom, blocked both the initial increase in metabolic rate as well as the subsequent rapid cell death seen at this concentration of venom alone. 10^{-4}M BRI2923, incubated with 100 μg/ml venom, also abolished the initial increase in metabolic rate and the cells proceeded to cell death after the venom/dendrimer solution was washed out. Snake venoms consist of

many toxic components each of which have different modes of toxicity. Cobra venoms contain a cytotoxic peptide which causes cell lysis in a way similar to bee venom toxin, melittin. The amino acid sequence of the cytotoxin isolated from *Naja malenoleuca* indicates that this toxin is highly basic (cationic) and would thus be readily inactivated by polyanionic compounds such as BRI2923. This electrostatic inactivation as a basis for reduced toxicity is supported by the experimental finding that an incubation period of 6 minutes gives the same result as the longer incubation periods of 30-60 mins used.

BRI2923 Inhibition of Melittin (major toxin from bee venom).

Melittin was added to the CEM cells in half log doses ranging from 10^{-5}M to 10^{-7}M. The two highest doses (10^{-5}M and 5 x 10^{-6}M) caused total cell death within 15 mins with no initial activation of the cells. 10^{-6}M caused a transient increase in cell metabolic rate followed by cell lysis, complete after approx. 1 hr. 5 x 10^{-7}M caused the lysis curve to shift further to the right and 10^{-7}M was without effect. This dose response determination was repeated and 10^{-6}M melittin was selected as the submaximal concentration to be used with BRI2923. A range of concentrations of BRI2923 were incubated with 10^{-6}M melittin for 20 mins. 10^{-4}M and 10^{-5}M BRI2923 completely inhibited the toxic effects of the melittin. 10^{-6}M BRI2923 completely blocked the melittin toxicity for approx. 30 mins and during the final 30 mins of exposure the metabolic rate only fell by approx. 10-15% less than the control cells. 10^{-7}M, 5 x 10^{-7}M and 10^{-8}M concentrations of BRI 2923 reduced, but did not prevent, toxicity. 10^{-9}M BRI2923 had no effect on 10^{-6}M melittin.

EXAMPLE 40

Inhibition of HIV (AIDS) Toxin

Introduction

Vpr is an Human Immunodeficiency Virus (HIV-1) accessory gene product, 14-kDa, 96-amino-acid protein. The gene *vpr* is highly conserved in HIV-1, HIV-2 and the Vpr gene product optimises HIV infection and disease progression. (reviewed by Cullen, 1998; Emerman & Malim, 1998). Among its actions, Vpr induces apoptosis and cytopathic effects in both human cells and yeast (Stewart et al, 1997; Zhao et al, 1996; Macreadie et al, 1996).

The Vpr protein has been fractionated and the peptide sequence which causes apoptosis has been isolated and designated Vpr P3. Vpr P3 has been found to permeabilise $CD4^+$ T lymphocytes (such as CEM cells) and causes their death by apoptosis. The following experiments use this toxic Vpr peptide fraction P3 (Arunagiri et al, 1997).

Method using the Cytosensor Microphysiometer

Human CEM cells were centrifuged at 1400 RPM for 7 minutes and resuspended in modified RPMI 1640 medium. Cells were then immobilised using an agarose entrapment medium and spotted onto the centre of the polycarbonate membrane of the capsule cup (Molecular Devices Ltd., CA.). The cells were seeded at a density of approximately 43,000 - 75,000 cells per capsule cup. The seeded capsule cups were transferred to sensor chambers containing the silicon sensor and positioned on to the microphysiometer. Modified media was pumped across the cells at a rate of 120μl/min. Each cell chamber was served by fluid from either of two reservoirs, which could be alternated using a software command. Cells were allowed to equilibrate within the chambers for 30-60 minutes or until a stable metabolic rate was

achieved. In this set of experiments, flow was stopped for 30 s, during which time, a least squares fit slope to the change in voltage signal over time, the acidification rate (measured as μV/s), was calculated. This rate data was normalised (using the 4-5 rate points prior to addition of compound) to allow direct comparison of the signals from the four chambers. Measurements of the acidification rate were made every 2 min. The chamber was held at 37°C.

For all experiments CEM cells were used at 1.3×10^5 to 1.3×10^6 cells/ml which corresponds to early to mid log phase growth of these cells.

The cells were set up on the microphysiometer and the Vpr P3 peptide was perfused through the cell chambers after an equilibration period of at least 30 minutes. The concentration range used for Vpr P3 was 10^{-5}M - 2×10^{-5}M . Vpr P3 needed to be in contact with the cells for upwards of 2 hours for the full effect of the toxicity to be apparent.

For experiments testing inhibition of Vpr peptide toxicity by BRI2923, media solutions of Vpr P3 peptides were made to the appropriate concentration and the BRI2923 was added, the solution mixed, and left to equilibrate for 20-30 minutes prior to placement on the cytosensor. With each inhibition experiment, a positive control channel containing the same concentration of the Vpr P3 used to test BRI2923 inhibition was also run for the same time period as well as a time control channel. The Vpr P3 peptide solution and the Vpr P3/BRI2923 solution were in contact with the cells for a minimum period of 2hr 30min to a maximum period of 4hr. Also, after washout of the compounds, the metabolic rate of the cells was monitored for a short time period (minimum of 6 min).

Results

In the cytosensor system, the VPR P3 peptide tested on its own, causes an initial increase in metabolic rate followed by a decline and subsequent cell death due to apoptosis of the CEM cells.

In the experiment shown in Figure 1, the VPR peptide P3 (at 10^{-5}M) was pre-incubated with the compound BRI2923 at 10^{-4}M (Final Volume) in a total volume of 1ml (modified RPMI media) for 30 minutes. Solutions were then diluted to final volumes (25ml) and perfused through the cells. A large initial drop in metabolic rate is seen in the BRI2923/Vpr P3 chamber, due to a combination of the pH difference of the 10^{-4}M BRI2923 solution (acidic) and the media (neutral) and an intrinsic buffering effect of the BRI2923 dendrimer itself. To account for this drop in metabolic rate, a correction factor has been applied to all results using BRI2923. The correction factor used was:

a) the calculated initial drop on the first data point added to all subsequent data points and

b) in the same way the calculated washout effect was subtracted from all points after the wash.

The corrected graph is shown in Figure 2. This result shows that BRI2923, at 10^{-4}M, gives complete protection against the toxic effects (apoptosis) induced by VPR P3 at 10^{-5}M. This experiment was repeated in quadruplicate.

Further concentrations of BRI 2923 were tested against higher concentrations of Vpr P3, and the results are shown in Figures 3 and 4. BRI 2923 at 10^{-5}M also demonstrated inhibition of $2x10^{-5}$M Vpr P3-induced apoptosis (experiment in triplicate). BRI2923 10^{-6}M showed a significant reduction in the effects produced by $2x10^{-5}$M Vpr P3 (triplicate experiment). 10^{-7}M BRI2923 on $2x10^{-5}$M Vpr P3 toxicity, attenuate to a lesser extent the rate of decline in metabolic rate (duplicate experiment).

Unlike the time controls the cells in the chamber containing BRI2923 did not show any time related decline in metabolic rate. Previous cytosensor experiments using BRI2923 have also suggested that the dendrimer may confer a cytoprotective effect in addition to the inhibition of apoptosis caused by VPR P3.

EXAMPLE 41

Calcein release assay to determine inhibition of cholera toxin.

Materials

Cell Line

- HPC-8 Human ileocecal carcenoma epithelial cells

Calcein, AM

- Molecular Probes, catalogue # C-1430
- $C_{46}H_{46}N_2O_{23}$, molecular weight 994.87
- Resuspend 1 mg vial in 100 µl reagent-grade, anhydrous dimethylsulphoxide (DMSO) to give a stock concentration of 10 mM.

Toxin

- Cholera Toxin (Sigma Cat. No. C8052) - A subunit surrounded by five B subunits.

Inhibitor Dendrimers

- Test dendrimers - BRI2913 and BRI2999.

Method

1. HCP-8 (adherent human ileocecal carcinoma epithelial cell line) in log phase growth were trypsinised (trypsin EDTA) in 2ml/75cm^2 flasks after 2 PBS (phosphate buffered saline) washes.
2. Cells with trypsin solution were incubated for 5 min.

3. 20ml culture medium added to flask to inactivate trypsin.
4. Cell solution transferred to 50ml centrifuge tube and spun at 1200 RPM for 7 min at room temperature (RT).
5. Cells washed in PBS and spun at 1200 RPM for 7 min at RT.
6. Cells resuspended in PBS and counted then spun at 1200 RPM for 7 min at RT and supernatant discarded.
7. Cells resuspended in 1ml PBS with 5µl calcein added and incubated at 37°C for 45 min.
8. Cells washed in PBS (approx. 5-20ml) and resuspended in PBS to give final numbers of $5x10^5$ cells/100µl.
9. 100µl cell solution ($5x10^5$ cells per well) added in each well used of a 96 well plate with 50µl of cholera toxin (dissolved in H_2O) and 50µl of dendrimer solutions (stock solution made to 10mM in DMSO and diluted in PBS). Each concentration repeated in triplicate.
10. Plate incubated for 2hr at 37°C in 5% CO_2 incubator.
11. Supernatant harvested and fluorometric analysis performed.

Results

Preliminary experiment performed. All results presented are the average fluorescence intensity of three replicates.

	Fluoresence Intensity (FI)	% inhibition of cholera toxin.
0.3mg/ml cholera toxin 100µM BRI2923 100µM BRI2999	72.1 66 59.5	0% 8.5% 17.5%
0.15mg/ml cholera toxin 100µM BRI2923 100µM BRI2999	65.8 61 59.4	0% 7.3% 9.7%
Spontaneous calcein Release (SR)	26.8	

% inhibition = 100 - (FI_{toxin} + dendrimer/FI_{toxin} x 100)

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